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A functional interferon regulatory factor-1 (IRF-1)-binding site in the upstream regulatory region (URR) of human papillomavirus type 16

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Abstract

We recently identified a new enhancer element (HIRE-1, HPV-Interferon Responsive Element-1) in the upstream regulatory region (URR) of human papillomavirus (HPV) type 16. HIRE-1 is located upstream from and in close proximity to the TATA box. HIRE-1 is 1 nt shorter in its 5′ sequence in comparison to a consensus IRF-1 binding site (IRF-E). Gel shift analyses clearly demonstrated that HIRE-1 is capable of binding IRF-1 in response to interferon-γ (IFN-γ) treatment. In a reporter system, HIRE-1 stimulated transcription in response to IRF-1 or IFNγ from both a heterologous or the homologous (p97) promoter in a dose-dependent manner. Mutations in the core binding sequence strongly decreased this enhancer activity. Interestingly, HIRE-1 stimulated transcription in the context of the full URR in a cell-type-specific manner, thereby suggesting the role of other cell-type-specific factors that might counteract with its function. Thus, our results may explain the inconsistent clinical and experimental results observed following IFN treatment of cervical lesions or cells. Also, this new enhancer may have an important function during inflammatory responses against HPV type 16.

Keywords: HPV16; Transcription; Promoter; Enhancer; Interferon; IRF-1; IRF-E

Introduction

Human papillomaviruses (HPVs) are small DNA viruses that have been found to be associated with cutaneous and mucosal benign and malignant lesions (Shah, 1998; zur Hausen, 1996). Studies confirmed that cervical carcinomas contain HPV type 16 in 50% of all patients (Bosch et al., 1995).

Cytokines such as TNF-α, IL-1, TGFβ1, or interferons (IFNs) have been shown to affect expression of several HPV types: this could be inhibition (De Marco et al., 1991; Johnson et al., 1999; Kyo et al., 1994; Nawa et al., 1990; Woodworth et al., 1990, 1992, 1996) or activation (Gaiotti et al., 2000; Kim et al., 2000; Woodworth et al., 1995, 1996). In fact, IFN treatment of patients with cervical intraepithelial neoplasia (CIN) led to mixed and controversial outcomes (Gross, 1997). Similarly, IFN treatment resulted in the differential regulation of HPV expression in various cervical cancer cell lines (Kim et al., 2000). IFNs initiate a series of phosphorylation events through the JAK/STAT signaling pathway, leading to activation of transcription factors such as STATs and IRF-1 (Darnell et al., 1994; Stark et al., 1998). STAT-containing complexes bind GAS (Decker et al., 1997) or IFNα-stimulated response element (ISRE) (Levy, 1998) elements, while IRF-1 binds IRF-E, and thus they regulate transcription of IFN-responsive genes (Taniguchi et al., 1995).

HPV transcription is controlled by elements present in the upstream regulatory region (URR) (Desaintes and De-

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Table 1
Putative ISRE/IRF-E sequences in the promoter region of HPV type 16

ISRE	GNGAAA.NN.GAAACT
E-IRS[a]	GNNRRAA.NN.GAAACY
IRF-E	GAAA.CC.GAAAC/G
HIRE-1	GTTG-AA.CC.GAAACC
HIRE-2	GCGTAA.CC.GAAATC

[a] R = A or G; Y = C or T (Iftner, 1990).

meret, 1997). The early (p97) promoter is located in the 3′ end of the URR, and, from this promoter, transcription of both the E6 and E7 genes is initiated. Binding sites for a number of cellular transcription factors that influence HPV transcription have been identified in proximity to the early promoter (O'Connor et al., 1995). However, our knowledge is very limited with respect to the association of those binding sites with interferon therapies, i.e., binding of IFN-induced transcription factors (STATs, IRFs, etc.) to the URR.

Accordingly, our aim was to identify functional IFN-induced, protein-binding element(s) in the URR of HPV type 16 and to study their effects on the transcription of HPV type 16.

Results

Search for putative IFN-induced, protein-binding sites in the URR region of HPV type 16

We used Gene Runner v.3.0 software (Hastings Software, Inc.) to analyze the genome of HPV type 16 for the presence of putative ISRE/IRF-E sites. We found two sequences that were located in proximity to the early promoter (Table 1) that were named HIREs (HPV Interferon-Responsive Elements). These sequences were partly predicted by Iftner (Iftner, 1990) and termed E-IRS.

Those HIRE sequences were located upstream from the TATA box: either in close proximity to it and designated as HIRE-1 (nt 48–57 for GAACCGAAAC) or farther from it and designated as HIRE-2 (nt 32–42 for GTAACCGAAAT). However, only HIRE-1 possesses the characteristic 3′ end (GAAAC), while HIRE-2 has a mismatch in its 3′ end (GAAA**T**C) compared to the consensus sequences (GAAAC). The 5′ region of HIRE-1 is one nucleotide shorter (GAA) than the consensus IRF-E (GAAA) sequence, while HIRE-2 has a nucleotide substitution in its 5′ sequence (G**T**AA). A doublet (CC) separated those two parts in all HIREs as in the consensus IRF-E (NN). Obviously, HIRE-1 exhibits the highest homology to the IRF-1-binding IRF-E consensus site.

HPV16 HIRE-1 is capable of binding IFN-γ-induced protein(s)

Because of the closest similarity to the consensus IRF-E, we used HIRE-1 for further analysis. Earlier we determined that HIRE-1 is capable of binding IFN-γ-induced proteins, but not IFN-α-induced protein(s), between 3 and 36 h post-treatment (Arany et al., 2001). Accordingly, HeLa cells were treated with IFN-γ (500 U/ml) for 3 h, and nuclear extracts were prepared. An electrophoretic mobility shift assay (EMSA) was carried out using these nuclear extracts and end-labeled, double-stranded oligonucleotides representing HPV 16 HIRE-1 or a consensus IRF-1 (c.IRF-1) binding sequence. Control, untreated HeLa nuclear extract was also used (Fig. 1, lane 2). Apparently, IFN-γ treatment induced binding of a protein complex to the HIRE-1 oligo that was similar to the binding to a consensus IRF-1 sequence (Fig. 1, lane 8). The specificity of this binding was determined by cold oligonucleotide competition: a 100-fold excess of either HIRE-1 (Fig, 1. lane 4) or a consensus IRF-1 oligonucleotide (Fig. 1, lane 5) outcompeted the binding. Also, 100-fold excess cold HIRE-1 was able to outcompete the protein bound to the consensus IRF-1 oligonucleotides (Fig. 1, lane 9). On the contrary, a double-mutated HIRE-1 (1T10T) was unable to compete with HIRE-1 for the IFNγ-induced protein (Fig. 1, lane 6) or was

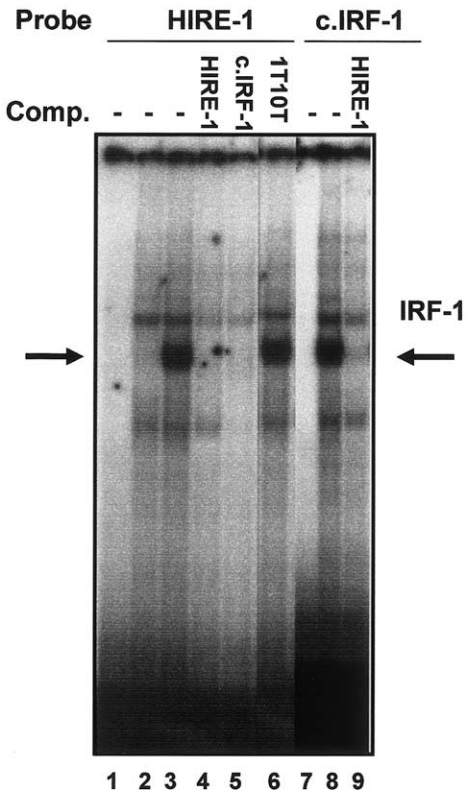

Fig. 1. HIRE-1 from HPV type 16 binds an IFNγ-induced protein. HeLa cells were treated with IFNγ for 3 h or left intact. Nuclear extracts were isolated and an EMSA assay was performed using an end-labeled HIRE-1 or a consensus IRF-1 (c.IRF-1) oligonucleotide. Left arrow indicates the position of the IFN-γ-induced band when HIRE-1 was used as a probe. The right arrow indicates the position of the IFN-γ-induced band (IRF-1) when c.IRF-1 was used as a probe. Competition experiments were performed using a 100-fold molar excess of cold oligonucleotides (Comp.) as listed. Lanes: 1 and 7, free probes; 2, untreated HeLa nuclear extract; 3–8, IFN-γ-treated HeLa nuclear extracts.

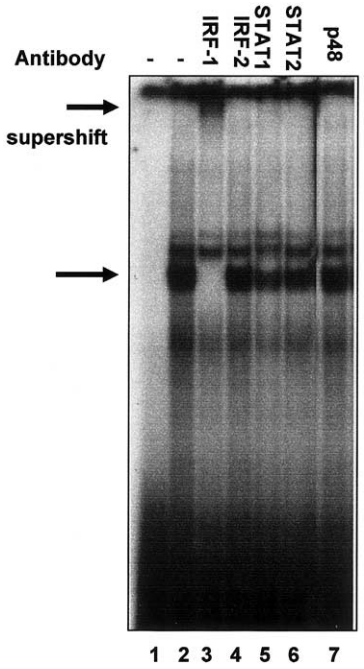

Fig. 2. IRF-1 binds HIRE-1. EMSA was performed using HIRE-1 probe and nuclear extracts from IFN-γ-treated HeLa cells. Supershift experiments were performed using antibodies directed against IRF-1, IRF-2, STAT1, STAT2, and ISGF3γ (p48). Lanes: 1, free probe; 2–6, IFN-γ-treated HeLa nuclear extracts.

unable to bind it (data not shown). Also, HIRE-2 did not bind any IFN-γ-induced protein (data not shown). It is interesting to note that nuclear extracts obtained from IFN-γ-treated A431, CX, or SiHa cells also showed the similar HIRE-1 binding pattern (Arany et al., 2001).

HIRE-1 binds IRF-1

We performed supershift assays to identify the nature of binding proteins in the complex that bound to the HIRE-1 after IFN-γ treatment (Fig. 2.). The IFN-γ-inducible band was supershifted/disrupted only by an anti-IRF-1 antibody, but not with antibodies against IRF-2, STAT1, STAT2, or ISGF3γ (p48).

IRF-1 induces transcription from the p97 or a heterologous promoter in a dose-dependent fashion

HeLa cells were cotransfected with a pSEAP–plasmid that contains a short segment of the URR from HPV type 16 fused to the SEAP reporter gene (pSEAP16sURR) plus an IRF-1 expression plasmid. This short segment spans a 96-bp fragment (nt 7–103) of the URR; it contains the TATA box, two E2Bs overlapping with 16HIRE-1, and the Sp1 binding site. Vector-transfected controls (SEAP-B) were also used. At 48 h after transfection the supernatants were analyzed for the presence of SEAP by a chemiluminescent method. As seen in Fig. 3A, IRF-1 significantly increased amounts of SEAP. In addition, cells were transiently cotransfected with a plasmid that contains three tandem repeats of HPV 16 HIRE-1 in front of enhancerless SV40 promoter fused to the SEAP reporter gene (wild type, pSEAP16–3xHIREwt, or mutant, pSEAP16–3xHIREmt) and an IRF-1 expression plasmid. At 48 h after transfection the supernatants were analyzed for the presence of SEAP. The amounts of SEAP significantly increased in the presence of the wild type, but not the mutant, 3XHIRE-1, when IRF-1 was cotransfected. These results suggested that HIRE-1 from HPV type 16 is a functional enhancer element that stimulates transcription both from the homologous (p97) promoter or from a heterologous promoter in response to IRF-1.

In another set of experiments, cells were transiently cotransfected with 2 μg of pSEAP16sURR (sURR) plasmid plus various amounts of IRF-1 expression plasmid, as indicated. SEAP activity was determined 48 h after transfection. Results are summarized in Fig. 4A. Apparently, SEAP

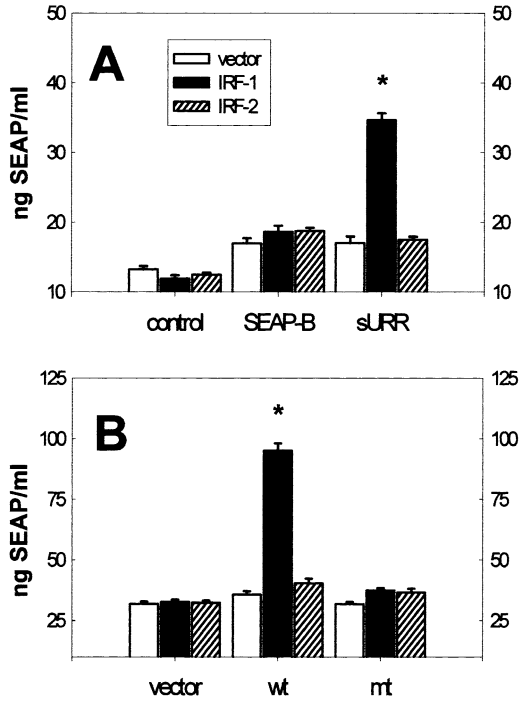

Fig. 3. IRF-1 induces reporter gene expression from the HPV early promoter or from a heterologous promoter. (A) HeLa cells were cotransfected with an IRF-1 or IRF-2 expression vector plus pSEAP16sURR (sURR) that contained the minimal promoter region of the HPV16 URR fused to the SEAP reporter gene. SEAP activity was monitored 48 h after transfection. Measurements also included transfection with the SEAP–Basic (SEAP-B) vector only. Transfections were done in triplicate. Values are given as median SEAP activities ± SEM. SEAP amounts were normalized to β-gal activity (see Materials and Methods). (B) HeLa cells were cotransfected with an IRF-1 expression vector plus a plasmid (pSEAP16–3×HIREwt or pSEAP16–3×HIREmt) that contained three copies of either the wild type (wt) or mutant (mt) HIRE-1 placed in front of an SV40 promoter and the SEAP reporter gene. SEAP activity was monitored 48 h after transfection. Transfections were done in triplicate. Values are given as median SEAP amounts per milliliter ± SEM. SEAP amounts were normalized to β-gal activity (see Materials and Methods).

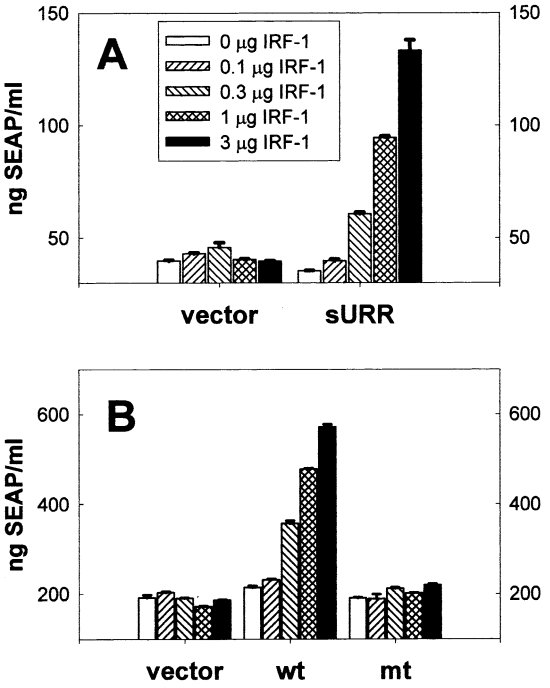

Fig. 4. IRF-1 induces SEAP activity in a dose-dependent fashion. HeLa cells were co-transfected with 2 μg of pSEAP16sURR (sURR) or pSEAP16–3×HIRE (wt or mt) plus indicated amounts of IRF-1 expression vector. SEAP activity was monitored 48 hours after transfection. Transfections were done in triplicate. Values are given as median SEAP amounts/ml.

amounts proportionally increased with the amounts of transfected IRF-1 plasmid. Similar responses were seen using the pSEAP16–3xHIREwt plasmid (Fig. 4B).

IRF-1 or IFN-γ increases the activity of the short URR in various cervical carcinoma cell lines

We transiently cotransfected several HPV-positive (HeLa, SiHa, CaSki and C4-II) and HPV-negative (A431, C33 and CX) cervical carcinoma cell lines with the short URR (pSEAP16sURR) plasmid plus an IRF-1 expression plasmid or with the pSEAP16sURR and treated the cell lines with IFN-γ for 48 h. SEAP activities were determined as described earlier. Results are shown in Fig. 5A and are given as fold changes compared to the vector-transfected values. Apparently, both IRF-1 and IFN-γ stimulated transcription from the short URR in all the cell lines we tested.

IRF-1 or IFN-γ affects the activity of the full URR in a cell-type-dependent manner in various cervical carcinoma cell lines

On the contrary, there were mixed results using the full URR in a similar setting (Fig. 5B). Transcription was increased in some cells (SiHa, A431, and C33A), but was unchanged (HeLa and C-4II) or even decreased (CX and CaSki) in the others. Apparently, this phenomenon did not reflect the presence or absence of endogenous HPV.

Discussion

Cytokines induce a variety of transcription factors in the target cells that play a role in their antiviral, antiproliferative, or immune modulatory activities. IFNs induce IFN-responsive genes such as IRFs (Nguyen et al., 1997; Taniguchi et al., 1997) through the JAK/STAT signaling pathway (Stark et al., 1998). IRF-1 serves as a transcription factor in mediating antiproliferative and antiviral activities of various cytokines (Taniguchi et al., 1995) and binds to a motif in the promoter of target genes, termed IRF-E, that has been determined to be GAAANNGAAAC (Taniguchi et al., 1997). IRF-1 also can bind to an element called IFNα-stimulated response element (ISRE), which is composed of direct repeats of the sequence GAAA spaced by two nucleotides (Levy, 1998). A common consensus for this sequence is GNGAAANNGAAACT (Taniguchi et al., 1997).

Our computer-assisted search identified two motifs in the URR of HPV type 16 that resembled IRF-E and/or ISRE elements (Table 1). Earlier, the existence of an ISRE-like element was suggested in the URR of several HPV types (Iftner, 1990). These sequences are located in close proximity to the early promoter (p97) and overlapped two E2Bs sites. The type found in the URR of HPV type 16 (HIRE-1,

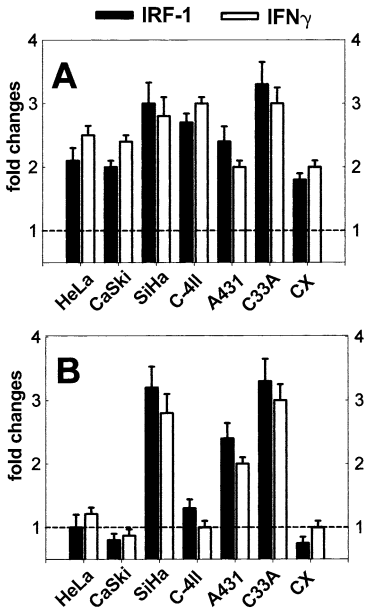

Fig. 5. Effects of IRF-1 or IFN-γ on the URR reporter gene activities in different cervical carcinoma cells. (A) Various (HPV-positive or -negative) cervical carcinoma cells were cotransfected with pSEAP16sURR and IRF-1 or pSEAP16sURR and treated with 500 U/ml IFNγ. SEAP amounts were detected 48 h after transfection, and values are given as fold changes compared to the vector-transfected and untreated controls (mean ± SD, $n = 3$). (B) Various (HPV-positive or -negative) cervical carcinoma cells were cotransfected with pSEAP16fURR and IRF-1 or pSEAP16fURR and treated with 500 U/ml IFNγ. SEAP amounts were detected 48 h after transfection and values are given as fold changes compared to the vector-transfected and untreated controls (mean ± SD, $n = 3$).

GAACCGAAAC) is closely homologous to the consensus IRF-E (GAAANNGAAAC) element (Table 1).

Closer analysis of HIRE-1 revealed that it binds IFN-γ-induced proteins in an inducible manner (Fig. 1.). Competition experiments demonstrated the specificity of this binding (Fig. 1). Mutations in this element abolished its competition for IRF-1 binding (Fig. 1, lane 6) and stimulation of the reporter gene in response to IRF-1 (Fig. 3A) or IFN-γ (Fig. 5).

The 5′ consensus-like sequence in HIRE-1 is a nucleotide shorter (GAA) than the consensus (GAAA) IRF-E, but it did not weaken the IRF-1 binding affinity of HIRE-1 (Fig. 1, lane 3) compared to the consensus sequence (Fig. 1, lane 8). Also, HIRE-1 was found to be a strong competitor to the consensus IRF-1 binding sequence as well (Fig. 1, lane 9).

Transient transfection experiments demonstrated that the HIRE-1 is a functional enhancer: it was able to stimulate transcription from the homologous (p97) and a heterologous promoter in response to IRF-1 or IFN-γ (Fig. 3 and Fig. 5A) in a dose-dependent manner (Fig. 4).

Interestingly, in the context of the full URR IRF-1, transfection or IFNγ treatment stimulated transcription from the p97 promoter in a cell-type-specific fashion (Fig. 5B) even though binding of IRF-1 to HIRE-1 didn't show this specificity (Arany et al., 2001). These results suggest that cell-type-specific factors that are related to IFN-γ or IRF-1 stimuli might counteract with the HIRE-1 stimulation. These observations are in agreement with those that described cell-type-specific silencing of HPV transcription (Sailaja et al., 1999). It is also known that the effects of IFNs on HPV early mRNA levels are highly diverse or even contradictory (Gross, 1997; Kim et al., 2000); therefore, cell-type specificity or status of the IFN-signaling pathway should be revisited.

In light of these facts, the exact role of HIRE in the regulation of HPV transcription remains partly unresolved. One can speculate that HIRE might function by influencing the overlapping E2 sites. Those E2 sites repress transcription from the HPV promoter, and thus, IRF-1-binding to the overlapping HIREs could be competitive or synergistic. It is tempting to hypothesize that HIRE might participate in the early phase of abolishment of immune surveillance by increasing E7 levels in response to inflammatory cytokines secreted by immune cells and thus might help to escape from immune surveillance. Either way, HIRE is a newly discovered enhancer element in the URR of HPV 16, which might be an important part of IFN responses during inflammation.

Materials and methods

Cell culture

HeLa, CaSki, SiHa, C-4II, A431, and C33A cells were purchased from ATCC and grown at 37°C in DMEM supplemented with 10% FCS and 100 μg/ml penicillin plus 100 U/ml streptomycin, in 5% CO_2 atmosphere. The HPV-negative CX cervical carcinoma cell line was a gift from Dr. Chen (Chou et al., 1996).

Plasmid construction

The minimal promoter region of HPV type 16 (nt 7–103) was inserted into a pSEAP2–Basic plasmid (pSEAP16sURR) as described earlier (Arany et al., 2002a). In a similar way a 1060-bp fragment of the HPV16 genome (between nt 7050 and 103) spanning the full URR plus the early promoter was constructed (pSEAP16fURR). The HPV16 genome was a gift from Dr. Teh-sheng Chan (Department of Microbiology and Immunology, UTMB, Galveston, TX).

The pSEAP16–3xHIREwt recombinant plasmid was constructed by annealing three copies of the wild type HPV 16 HIRE-1 (5′-GTTGAACCGAAAC-3′) with linker sequences (5′-*Kpn*I, GGTACC, and 3′-*Bgl*II, AGATCT) into a pSEAP2–promoter plasmid (Clontech, Palo Alto, CA). The pSEAP16–3xHIREmt was constructed by annealing three copies of the mutated HPV 16 HIRE-1 (5′-GTT**T**A-ACCGAAA**T**-3′) with similar linker sequences into a pSEAP2–promoter plasmid (Clontech, Palo Alto, CA).

Construction of the IRF-1 expression vector was described earlier (Arany et al., 2002a). The IRF-1 cDNA was a gift from Dr. Taniguchi (Department of Immunology, Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Japan).

Electrophoretic mobility shift assay

Nuclear extracts were prepared by lysing cells in a buffer containing 10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM DTT, 1 mM EDTA, 0.5% NP-40, and 1 mM PMSF. Five to ten micrograms of nuclear extracts was incubated in a binding buffer (10 mM Tris–HCl, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 6% glycerol, 1 mM DTT, and 1 μg polydIC) in the presence or absence of a 100-fold molar excess of unlabeled competitor DNA and/or appropriate antibodies on ice for 10 min. Following incubation, 10,000 cpm of ^{32}P end-labeled oligonucleotide probe was added and the reaction incubated at room temperature for an additional 30 min. The DNA–protein complexes were separated from the free probe by electrophoresis on a 6% polyacrylamide gel. The gel was dried and subjected to autoradiography. Oligonucleotides were custom designed and synthesized (Bio-Synthesis, Inc., Denton, TX). Annealing of individual oligos was done according to standard protocols.

Gel-shift oligonucleotides

For gel-shift competitions, oligonucleotides were synthesized by Bio-Sysnthesis, Inc. (Denton, TX), and were annealed according to a standard protocol. These oligos are

listed below. The putative core binding sequence is underlined; mutated bases are in bold (the sense strand is shown):

HPV16 HIRE-1	ATCGGTTGAACCGAAACCGG
1T10T HIRE-1 mutant	ATCGGTT**T**AACCGAAA**T**CGG

The consensus IRF-1 gel-shift oligonucleotide (GGAAGCGAAAATGAAATTGACT) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Plasmid transfection

HeLa cells were transfected with the above described or control plasmids using GenePORTER 2 transfection reagent (GTS Inc., San Diego, CA) as reported earlier (Arany et al., 2002a, 2002b). Aliquots of the supernatants (100 μl) were removed at various time points (24 and 48 h, respectively) and kept at −70°C until SEAP activity was measured.

Measurement of SEAP reporter activity

The pSEAP2 plasmids (Clontech) used SEAP (a secreted form of human placental alkaline phosphatase) as a reporter gene. Aliquots of 100 μl from the supernatants were removed and used for SEAP determinations (Arany et al., 2002a, 2002b).

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