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# Interleukin-10 induces transcription of the early promoter of human papillomavirus type 16 (HPV16) through the 5′-segment of the upstream regulatory region (URR)

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**Abstract**

The effects of various proinflammatory cytokines on the transcription of human papillomaviruses (HPVs) have been demonstrated. On the other hand, the role of anti-inflammatory cytokines has not been elaborated, despite the fact that levels of interleukin-10 (IL-10) have been found significantly elevated in cervical dysplasias or carcinomas as well as in the cervix of HIV-positive individuals. These conditions are also associated with elevated viral transcription. Thus, the impact of IL-10 on HPV transcription might be important in pathogenesis of cervical lesions in both immunocompetent or immunosuppressed individuals. In this paper we describe the effects of IL-10 on the transcription of HPV type 16. We found that treatment of HPV 16-positive cervical carcinoma cells with IL-10 increased mRNA levels of the E7 early gene at the level of transcription. Similarly, IL-10 significantly and dose-dependently induced the transcription from the HPV early promoter in a reporter system. Employing deletion mutants we determined that this induction is mapped to the 5′ segment of the URR. Transient transfection of an antisense-STAT3-expression vector abolished IL-10-induced reporter activity as well as HPV 16 E7 expression. This suggests that STAT3 either directly binds to the URR and stimulates transcription or affects expression and/or binding of transcription factors that bind to the 5′-region. Our findings suggest a mechanism by which—in addition to its immunosuppressive effects—IL-10 might enhance persistence and progression of HPV-related lesions under conditions (e.g. dysplastic progression, HIV infection) when the cytokine expression in the cervical microenvironment changes. 

*Keywords:* HPV; Transcription; Promoter; IL-10; STAT3

## 1. Introduction

Cytokines are important regulators of HPV transcription (Khare et al., 1995; Kyo et al., 1994): inflammatory cytokines, such as IFN-α, IFN-γ, IL-1, TNF-α and IL-6 inhibit mRNA levels of HPV genes (Bauknecht et al., 1999; De Marco et al., 1991; Kyo et al., 1994; Nawa et al.,

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1990; Woodworth et al., 1996). On the other hand, the effects of anti-inflammatory cytokines on HPV transcription are completely unknown despite their elevated expression under various conditions. Cervicovaginal washings from patients with cervical cancers contain higher amounts of IL-10 than washings from healthy donors or patients with cervical dysplasias (Tjiong et al., 2001). Also, expression of IL-10 is significantly higher in the cervical epithelium from patients with cervical dysplasias than the normal cervix (Clerici et al., 1997; El-Sherif et al., 2001; Mota et al., 1999). In addition, the expression of $Th_2$-type cytokines, such as IL-4, IL-5 or IL-10 is significant in the cervical mucosa and in the cervical mucus during HIV infection (Crowley-Nowick et al., 2000; Olaitan et al., 1998; Sha et al., 1997). It has been suggested that these changes might represent changes in the immune status of the local cervical microenvironment that determines persistence of HPV and progression of anogenital dysplasias into invasive cancer (El-Sherif et al., 2001). The mechanisms by which IL-10 might influence tumor growth are unknown. IL-10 could downregulate the expression of the major histocompatibility complex (MHC) type I and prevent CD8-dependent tumor antigen presentation (Matsuda et al., 1994). Also, IL-10 could down-modulate CD4-dependent antigen presentation and MHC II expression (Moore et al., 1993). On the other hand, IL-10 enhanced growth of HPV-18-immortalized high-grade prostatic intraepithelial neoplastic cells in vitro (Wang et al., 1999). These findings raise the possibility of a regulation pattern other than immune regulation: the effects of IL-10 on HPV transcription.

Accordingly, our aim was to determine the effects of IL-10 on HPV type 16 transcription.

## 2. Materials and methods

### 2.1. Cell culture

HeLa and SiHa cells were purchased from ATCC and grown at 37 °C in DMEM supplemented with 10% FCS in a 5% $CO_2$ atmosphere.

### 2.2. Real-time quantitative RT-PCR

We determined mRNA levels of HPV 16 E7 by a one-step real-time RT-PCR procedure using the TaqMan EZ RT-PCR kit (Perkin–Elmer, Foster City, CA) with an ABI Prism 7700 sequence detector (Perkin–Elmer) as published by Choo et al. (2000). Experimental conditions and primers were the same as published. We determined the cycle threshold ($C_T$) value, i.e. the cycle number at which a significant increase in the fluorescence signal was first detected. HPV16 E7 mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Fold increase (mean ± S.D.) was compared to the untreated controls.

### 2.3. Nuclear run-off assay

Nuclei were isolated from the appropriate cells using approximately $1–2 \times 10^8$ cells for one experiment (3xT175 flasks). The run-off was carried out at 30 °C for 30–60 min in a buffer containing 1 mM ATP, CTP, GTP and 200 μCi [α-$^{32}$P]UTP as described earlier (Arany et al., 1992). The transcripts were hybridized to cDNAs immobilized to Hybond N+ membrane (Amersham, Piscataway, NJ). After hybridization, filters were washed, treated with 10 μg/ml RNase for 30 min and exposed to Hyperfilm MP (Amersham, Piscataway, NJ).

### 2.4. Preparation of cell lysates and Western blotting

Cultured cells were lysed at 4 °C in RIPA (0.3 ml/T75 flask) supplemented with 10 mg/ml PMSF plus 1% protease inhibitor cocktail (Sigma). Protein content was determined with a "BioRad $D_C$ Protein Assay" (BioRad, Hercules, CA) method that allows the presence of high detergent concentration. 50 μg of total cell lysate was subjected to SDS/PAGE electrophoresis (Protean Minigel system, BioRad, Hercules, CA) and the proteins were blotted to a polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules, CA). The membranes were blocked in a Tris-buffered sodium chloride-Tween 20 (TBST) solution and incubated with primary antibodies, according to standard

protocols, then the blots were probed according to the ECL Plus Western blotting protocol (Amersham, Piscataway, NJ), using 1:10 000 diluted HRP conjugated secondary antibody (Santa Cruz Immunochemicals, Santa Cruz, CA). Anti-STAT3 and antiphosphoSTAT3 (clone 9E12) antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-IL10-receptor antibody (clone 3F9) was purchased from BD Pharmingen (Franklin Lakes, NJ). The HPV16 E7 antibody was purchased from Santa Cruz Immunochemicals (clone 9E12)

### 2.5. Plasmid construction

Three different fragments of the HPV16 URR were obtained by PCR from a cloned HPV 16 plasmid (gift from Dr. T-s. Chan, UTMB). These fragments span the full URR (nt7050-103), the central- plus 3′-segments (nt7458-103) or the 3′-segment (nt7-103) and were inserted into a pCR2.1 plasmid (Invitrogen, Carlsbad, CA). These plasmids were cut with KpnI/XhoI enzymes, and the resultant fragments were inserted into a pSEAP2-Basic vector in front of the SEAP (secreted alkaline phosphatase) reporter gene (Clontech, Palo Alto, CA) using common molecular cloning techniques.

### 2.6. Plasmid transfection

HeLa cells were transfected with the above described or control plasmids using GenePORTER 2 transfection reagent (GTS Inc., San Diego, CA). Transfections were made in 60-mm Petri dishes. Cells were plated at about $5 \times 10^5$ cells/dish density in 2.5 ml of culture medium 24 h before the transfection to ensure that they were 60–90% confluent on the day of transfection. Then, 1 μg of plasmid DNA was diluted and incubated at room temperature for 10 min and then added to the plates. After 24 h fresh medium was added; transfections were made in triplicate. Controls, such as mock-transfected and vector-transfected cells, were included. 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.

### 2.7. Measurement of SEAP reporter activity

The pSEAP2 plasmids (Clontech, Palo Alto, CA) used SEAP (a secreted form of human placental alkaline phosphatase) as a reporter gene. Aliquots of 100 μl from the supernatants were removed and spun down to remove particles. To each 25-μl sample, 75 μl of dilution buffer was added and the mixture incubated at 65 °C for 30 min to inactivate endogenous AP activities. After cooling, 100 μl assay buffer was added to the samples and incubated for 5 min at room temperature. Then, 100 μl of diluted chemiluminescent substrate CSPD was added to each sample, which was incubated at least 10 min at room temperature. Readings were done 10–60 min after substrate addition. The reactions were performed in 96-well plates, and signals were detected and recorded in a ChemiImager (Alpha Innotech, San Leonardo, CA). The AlphaEase 4.0 software allowed us to use the 96-well plate format for detection and to quantitate the results.

### 2.8. Measurement of transfection efficiency

The CMV-promoter-driven β-galactosidase (β-gal) plasmid was purchased from Clontech (Palo Alto, CA) and was co-transfected with the above-mentioned plasmids (1 μg per dish). The β-gal activity was measured by using the Galactron-Star chemiluminescent substrate (Clontech, Palo Alto, CA). After 48 h of transfection supernatants were collected for the SEAP activity, cells from each dish were scraped and washed in PBS. Cells were lysed with a potassium-phosphate-based solution and aliquots were combined with a reaction buffer according to the manufacturer's recommendation. The reaction was exposed on a white Xenobond plate in a ChemiImager (Alpha Innotech, San Leonardo, CA) by procedures similar to those for the SEAP assay. SEAP results were corrected with β-gal activities.

### 2.9. Statistical analysis

Statistical significance between the treated and control groups were determined by the appropriate *t*-test. Differences between means were con-

sidered significant if $P < 0.05$. All analyses were performed using a SigmaStat 2.01 software package.

## 3. Results

### 3.1. Effects of IL-10 on the expression of HPV16 E7

HPV16-positive cervical carcinoma cells (SiHa) were treated with 50 ng/ml of recombinant human IL-10 (R&D, Minneapolis, MN) for 24 h. mRNA levels of HPV16 E7 were determined by a real-time PCR method. IL-10 increased HPV16 E7 mRNA levels moderately, but significantly (Fig. 1A.). This effect is due to an increase in transcription rate as determined by nuclear run-off assay (Fig. 1B). A similar increase in E7 mRNA levels in other HPV16-positive cell lines was also induced by IL-10 (Fig. 1A). Protein levels of HPV16 E7 were also increased after IL-10 treatment (Fig. 1C).

A

| | mean | SD |
|---|---|---|
| SiHa | 2.3* | 0.5 |
| CaSki | 2.5* | 0.65 |
| HKc16 | 2.8* | 0.48 |

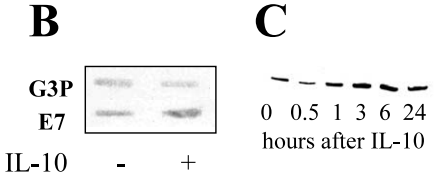



Fig. 1. Effects of IL-10 on HPV16 E7 expression. (A) SiHa cells were treated with 50 ng/ml of IL-10 for 24 h. mRNA levels of HPV16 E7 were determined by real-time PCR. Results are shown as mean fold increase of three independent experiments. (* $P = 0.005$ by nonpaired $t$-test) In addition, HPV 16-positive cervical carcinoma cells (CaSki) or HPV 16-immortalized human keratinocytes (provided by T.-S. Chan) were tested in a similar manner. (B) Nuclear run-off assay was done using nuclear extracts from IL-10 treated (+) or untreated (−) SiHa cells. The method is described in Section 2. (C) SiHa cells were treated with 50 ng/ml of IL-10 for the time indicated. Western blotting was performed to detect HPV16 E7 proteins.

### 3.2. Functional analysis of the HPV16 URR: identification of an IL-10 responsive region

The URR of HPV16 contains three distinct regions (O'Connor et al., 1995) the 5′-, the central- and the 3′-segments, respectively. These segments bind different sets of transcription factors and thus, affect the transcription from the early (p97) promoter. We cloned the full-length URR, the central- plus 3′-segments and the 3′-segment into a pSEAP2-Basic reporter plasmid (Fig. 2A). These reporter plasmids were transiently transfected into HeLa cells together with a CMV-driven β-galactosidase (β-gal) plasmid overnight and then treated with 50 ng/ml of IL-10 for an additional 24 h. SEAP amounts were determined 48 h after the transfection by a chemiluminescent method. SEAP amounts were increased moderately, but significantly only from the full-length URR (Fig. 2B), which suggested that the 5′-segment of the URR is responsive to IL-10.

The effect of IL-10 on the URR was dose-dependent (Fig. 3A); increasing IL-10 concentration (up to 100 ng/ml) resulted in increased SEAP secretion. We also analyzed the effects of IL-10 in HPV-negative cervical carcinoma cells, such as C33A and CX (Fig. 3B.). IL-10 did not change SEAP levels in C33A or CX cells. Also, SEAP levels in CX cells were significantly lower than in the other cell types. Considering these results and the fact that HeLa cells are commonly used for transfection in the literature, we used them in further experiments despite the fact that they harbor integrated copies of HPV type 18.

### 3.3. IL-10 and JAK/STAT signaling in cervical carcinoma cells

IL-10 utilizes the JAK/STAT signal transduction pathway through inducing STAT1 and STAT3 transcription factors in mononuclear cells (Donnelly et al., 1999; Riley et al., 1999). By a RT-PCR method we could detect mRNA for the IL-10 receptor (Fig. 4A) in SiHa cervical carcinoma cells, although at significantly lower levels than in PBMCs. Also, IL-10-induced SEAP activity was abolished by adding an anti-IL-10-receptor antibody (Pharmingen) prior to IL-10 treatment (Fig.

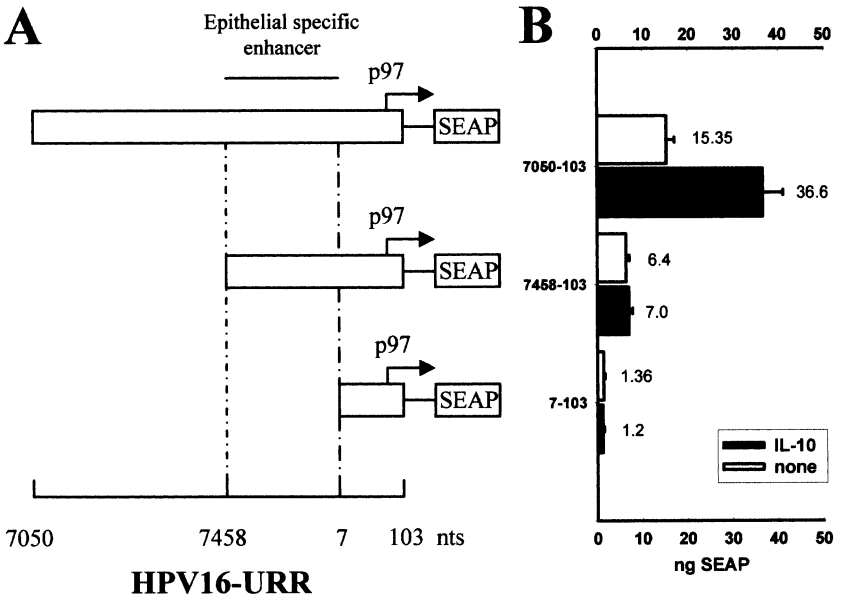



Fig. 2. A schematic representation of reporter plasmid constructs and the results of transient expression assays. (A) Various lengths of HPV16 URR fragments were linked to the SEAP reporter gene. (B) Various reporter plasmids were contransfected with a β-galactosidase (β-gal) expression plasmid in HeLa cells overnight, then treated with 50 ng/ml of IL-10 for 24 h. Aliquots from the supernatants were removed 48 h after transfection and analyzed for SEAP. Cell lysates were assayed for β-gal. Results are given as the mean of ng/ml secreted SEAP ± S.D. Mean values are given next to each bar.

4B). These data suggested that the IL-10 receptor is expressed and functions in cervical carcinoma cells. Western blotting determined that IL-10 increases phosphorylation of STAT3 but not STAT1 (Fig. 4C).

### 3.4. IL-10 regulates HPV16 E7 transcription through STAT3

An antisense STAT3 (AS-STAT3) expression vector (Grandis et al., 2000) was transiently transfected into HeLa cells together with the full-length URR-containing SEAP vector. Cells were treated with IL-10 and SEAP activity was determined (Fig. 5A). The AS-STAT3 transfection completely abolished IL-10-induced SEAP induction and also abolished constitutive SEAP expression. In addition, AS-STAT3 diminished E7 mRNA induction after IL-10 treatment (Fig. 5B). These results suggest a role of STAT3 in regulation of transcription from the p97 early promoter.

## 4. Discussion

HPV transcription is regulated through the URR of the HPV genome (Bernard and Apt, 1994) by positive and negative cellular regulatory proteins; the overall balance between these factors determines the final outcome of HPV promoter activity. The URR is divided into three functionally distinct segments, which are called the 5′, the central, and the 3′ segment (O'Connor et al., 1995).

The 5′ segment is about 300 bp in size and bracketed by the termination codon of L1 and an E2 binding site. This segment contains transcription termination and polyadenylation sites for late transcripts as well as a negative regulatory element acting at the level of late mRNA stability (Kennedy et al., 1991). The function of this region in regulating viral expression of HPVs, however, has been largely uncharacterized. This might be due to the fact that the impact of the 5′ segment on the viral early promoter is significantly smaller than that of the central segment (Kanaya et al., 1997).

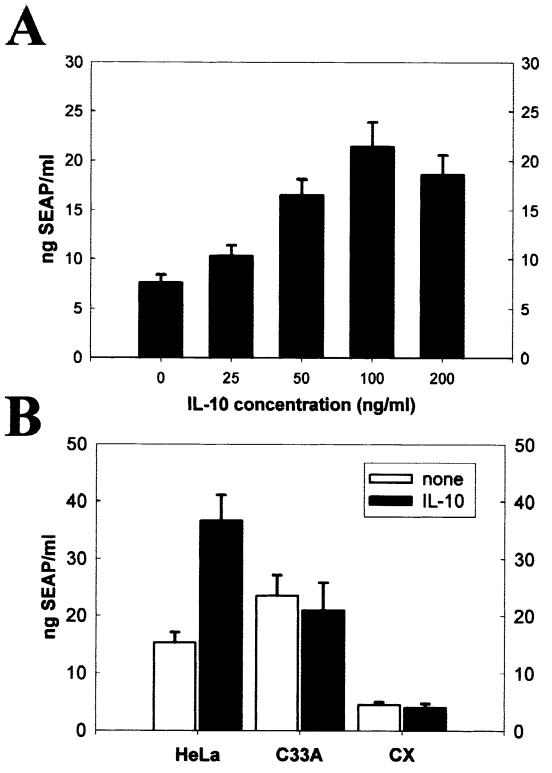



Fig. 3. Dose- and cell line-dependent induction of SEAP by IL-10. (A) The reporter plasmid containing the full-length URR of HPV16 was cotransfected with a β-galactosidase (β-gal) expression plasmid in HeLa cells overnight, then treated with the indicated concentrations of IL-10 for 24 h. Aliquots from the supernatants were removed 48 h after transfection and analyzed for SEAP. Cell lysates were assayed for β-gal. Results are given as mean of ng/ml secreted SEAP±S.D. (B) HeLa, C33A or CX cervical carcinoma cell lines were transiently transfected with the reporter plasmid containing the full-length URR of HPV16 and treated with 50 ng/ml of IL-10 for 24 h. Aliquots from the supernatants were removed 48 hours after transfection and analyzed for SEAP. Results are given as mean of ng/ml secreted SEAP±S.D.

Our experiments on HPV type 16 URR also supported this observation; the constitutive expression of the full URR was diminished to a lesser extent after deleting the 5′ segment than the central segment (Fig. 2B). Moreover, these data also demonstrated a role of the 5′ segment in constitutive activation of the early promoter. In the 5′URR of the HPV-16 one YY1 (nt: 7438), one Tef-1 (nt: 7189) and one AP-1 (nt: 7306) sites have been described by (O'Connor et al., 1995). In the 5′URR of HPV-11 a single copy of a 10-bp motif doublet is sufficient for enhancer function and this motif binds a 41-kDa protein (Auborn and Steinberg, 1991). Several binding sites for the differentiation-specific factor CDP/Cut that represses transcription have been reported in the 5′URR of the HPV-16 (O'Connor et al., 2000). In the 5′URR of HPV-31, three YY1 binding sites were identified that exhibited enhancer activity on both heterologous and homologous promoters (Kanaya et al., 1997). Interestingly, HPV-16 contains only one YY1 site in the corresponding region, but its function has not been identified.

Two E2 sites flank the central segment and it is known in HPV type 16 as the epithelial specific enhancer (Cripe et al., 1987). At least 11 different cellular transcription factors have been shown to bind in vitro to more than 20 different sites of the HPV-16 enhancer (Bernard and Apt, 1994). The 3′ segment contains the origin of replication and the early (p97) promoter that contains a TATA box, one Sp1 and two E2 binding sites. These four sites are complex means for modulating E6/E7 promoter activity (O'Connor et al., 1995).

Our studies showed that IL-10 induces transcription of HPV16 E7 modestly, but significantly (Fig. 1A–B). This IL-10 responsiveness is mapped to the 5′-segment of the URR (Fig. 2B).

IL-10 is known to modify the expression or binding affinity of transcription factors that are induced by proinflammatory cytokines (Dokter et al., 1996). However, our knowledge of associations between cytokine-driven activity (especially IL-10) of transcription factors and the HPV promoter is very limited. IL-10 activates a diverse array of functional responses in macrophages, B- or T-cells. IL-10 utilizes the JAK/STAT pathway in activating several STAT proteins, such as STAT1, STAT3 or STAT5 (Donnelly et al., 1999; Wehinger et al., 1996). Although IL-10 has pleiotropic effects on a variety of different cell types, its effects on epithelial keratinocytes are not known. Keratinocytes do not express IL-10, but its expression could be induced through CD23 ligation (Becherel et al., 1997) or by UV radiation (Enk et al., 1995; Grewe et al., 1995), even though this is a matter of debate (Jackson et al., 1996). Keratinocytes express functional IL-10 receptors (Michel et al.,

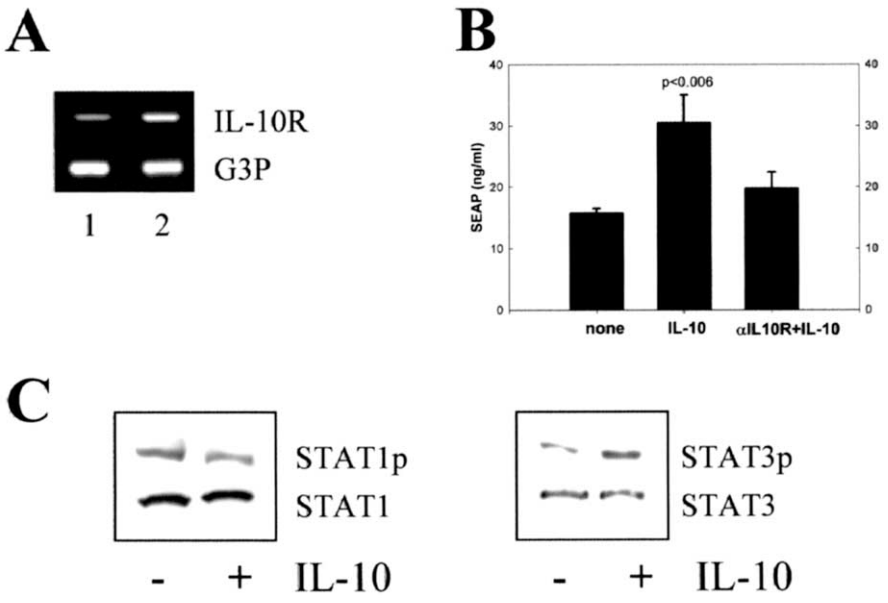
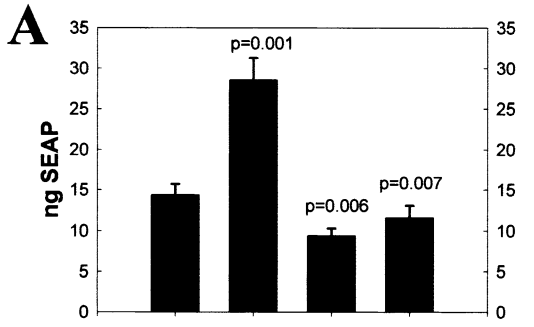



Fig. 4. IL-10 signaling in cervical carcinoma cells. (A) A semiquantitative RT-PCR method was employed to detect mRNA of IL-10-receptor in untreated SiHa cells (1) or human PBMCs (2). Glyceraldehyde-3-phosphate-dehydrogenase (G3P) was also amplified in order to demonstrate equal RNA loading. (B) HeLa cells were transiently cotransfected with a reporter plasmid containing the full-length URR of HPV16 and a β-galactosidase (β-gal) expression plasmid overnight, then treated with 50 ng/ml IL-10 for 24 h. In another setting, 10 ng/ml of anti-IL-10-receptor antibody was added to the cells 1 h before IL-10 treatment. Aliquots from the supernatants were removed 48 hours after transfection and analyzed for SEAP. Cell lysates were also assayed for β-gal. Results are given as mean of ng secreted SEAP ± S.D. Significance compared to the control was tested with a paired *t*-test. (C) SiHa cells were treated with 50 ng/ml of IL-10 for 1 h. Phosphorylation status of STAT1 and STAT3 along with STAT1 or STAT3 expression was determined by Western blotting.

1997), but the IL-10 signaling mechanism in epithelial keratinocytes is still unknown.

Our experiments showed that cervical keratinocytes express IL-10-receptor mRNA (Fig. 4A), although at significantly lower levels than do PBMCs. This observation might explain the requirement for relatively higher amounts of IL-10 and the modest impact on HPV transcription. These receptors are functional, because (a) IL-10 affects HPV transcription in a dose-dependent manner (Fig. 3), and, (b) adding an anti-IL-10-receptor antibody significantly abolished IL-10-induced induction of SEAP activity (Fig. 4B). Furthermore, IL-10 induced phosphorylation of STAT3 without affecting STAT3 levels (Fig. 4C). Apparently, this STAT3 phosphorylation is intimately related to the stimulatory effects of IL-10 on HPV16 transcription: applying an antisense STAT3 vector knocked-out the IL-10-induced increase in SEAP activity (Fig. 5A) and E7 induction (Fig. 5B). Our results are in agreement with the observation published by Smola-Hess et al. (2001). They reported that a dominant-negative STAT3 but not STAT1 blocks IL-6-mediated activation of the HPV 18-LCR in vitro. Since the IL-10-responsive site(s) is mapped to the 5′-segment of HPV16 URR, we should consider the following: (a) the 5′-segment contains STAT3-binding site(s), and/or, (b) IL-10 affects transcription factors that bind the 5′-segment, via induction of STAT3. Identification of these factors is in progress.

Our findings suggest a mechanism by which—in addition to its immunosuppressive effects—IL-10 might enhance persistence and progression of HPV-related lesions under conditions (e.g. dysplastic progression, HIV infection) when the cytokine expression in the cervical microenvironment changes.




| IL-10 | none | yes | yes | none |
|---|---|---|---|---|
| AS-STAT3 | none | none | yes | yes |

B

| | mean | S.D. |
|---|---|---|
| IL-10 | 2.36 | 0.4 |
| AS-STAT3+IL-10 | 0.92* | 0.23 |

Fig. 5. Effects of antisense STAT3 on HPV16 transcription. (A) The reporter plasmid containing the full-length URR of HPV16 was cotransfected with a β-galactosidase (β-gal) expression plasmid as well as with an antisense-STAT3 (AS-STAT3) expression plasmid in HeLa cells overnight, then treated with the indicated concentrations of IL-10 for 24 h. Aliquots from the supernatants were removed 48 h after transfection and analyzed for SEAP. Cell lysates were assayed for β-gal. Results are given as the mean of ng secreted SEAP ± S.D. Statistical analysis was performed using a paired *t*-test. (B) SiHa cells were treated with 50 ng/ml of IL-10 for 24 h in the presence or absence of AS-STAT3. HPV16 E7 mRNA levels were determined by real time RT-PCR. Results are shown as mean fold increase of three independent experiments. * $P = 0.009$ compared to the IL-10-treated samples (nonpaired *t*-test).

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