*Proc. Indian natn Sci Acad.* B69 No.1 pp23-34 (2003)

# Cellular Control of Human Papillomavirus Gene Activity in Cervical Cancer

B C DAS

*Division of Molecular Oncology, Institute of Cytology & Preventive Oncology (ICMR)*
*Maulana Azad Medical College Campus, New Delhi-110002*

(Received on 2 August 2001; Accepted after revision 22 February 2002)

Specific types of high risk human papillomaviruses(HPVs), particularly the HPV type 16 and HPV 18 are known to cause cervical cancer in women. Constitutive expression of two early genes, E6 and E7 of these HPVs which are responsible for tumorigenic transformation, is dependent mainly on the availability of host-cell transcription factor AP-1 that plays a key role during development of cervical cancer. Different oncoproteins such as, c-jun, jun-B, jun-D, c-fos, fos-B, fra-1 and fra-2 can either homo- or heterodimerize to form a functional AP-1 which binds to cognate DNA sequences within the viral upstream regulatory region.

In order to understand the involvement of redox regulatory pathways in the expression of HPVs, HPV positive cells were treated with a potent antioxidant, pyrrolidine dithiocarbamate (PDTC). It selectively suppressed the viral gene expression at the level of initiation of transcription but induced an enhanced binding of AP-1. Molecular dissection of AP-1 complex in electrophoretic mobility supershift assays (EMSAs) using antibodies raised against AP-1 family members showed that the normal composition of AP-1 consisting of c-jun homodimer was altered. c-jun became phosphorylated and heterodimerized with Fra-1 instead of its canonical dimerization partner c-fos. To address the question whether alteration in AP-1 composition could be responsible for a negative regulatory pathway controlling HPV transcription, different cell lines, such as HeLa-fibroblast non-tumerigenic hybrid cells 444, their highly tumorigenic segregants CGL3 and parental HeLa cells, all positive for HPV 18, were used as experimental models. AP-1 composition was found to differ considerably between these cell lines: c-fos was found to express in high quantity in tumorigenic cells HeLa and CGL3 while it is completely absent in non-tumerigenic 444 cells indicating crucial role of c-fos in tumorigenic transformation. An enhanced ectopic expression of c-fos gene in nontumorigenic 444 cells caused a change in the Jun/Fra-1 heterodimerization towards jun-fos which is detected in CGL3 and HeLa cells and the cells became tumorigenic. These results unravel a novel role of AP-1 transcription factor as an indispensable component controlling transcription of pathogenic HPV through alteration of AP-1 composition. Furthermore, the antioxidant-induced selective suppression of HPV expression provides a clue for a possible development of a novel therapeutic approach to control HPVs by antioxidative drugs.

Key Words: **Human papillomavirus (HPV), AP-1, Cervical cancer, URR, Jun, Fos, Fra-1, Transcription regulation, Antioxidant, Pyrrolidine dithiocarbamate**

## Introduction

Gene expression is controlled by the interplay of sequence-specific DNA binding proteins or transcription factors, which, in some cases, may be the targets of signal transduction pathways. Several protooncogenes such as c-myc, c- myb, c-fos, and c-jun encode sequence-specific transcription factors. Generally, the activity of these transcription factors can be modulated by phosphorylation or dephosphorylation (Hunter & Karin, 1992) but recent studies indicate that there exists an unusual form of regulation of DNA binding activity mediated by changes in reduction-oxidation (redox) status of the cells. For example, activator protein-1 (AP- 1) which is formed either by a homodimer of two jun proteins or a heterodimer of jun and fos proteins plays an important role in controlling expression of genes involved in cellular

*E-mail: bcdas48@hotmail.com, Fax: 011-23233406*

proliferation, differentiation and neoplastic transformation (see review by Angel & Karin 1991). The binding of these proteins to DNA requires that these proteins should be in a reduced state. This type of redox regulation may be wide spread because the DNA binding activities of several other transcription factors such as myb, rel and NFkB are also sensitive to changes in cellular oxidation state. Recently, AP-1 has been shown to play a pivotal role in transcriptional regulation of human papillomavirus (HPV) oncogene expression, which is essential for tumorigenic transformation of cervical epithelium.Human papillomaviruses (HPVs) are a group of small DNA tumour viruses that cause a variety of benign epithelial lesions and cervical and other neoplasia in humans. Constitutive expression of E6 and E7 ORFs (open reading frame) of specific types of "high risk" HPVs such as HPV type 16 and HPV 18 which are considered to be the principal causal agents for the development of cervical cancer in women is dependent mainly on the availability of AP-1 transcription factor of the host cells. This is evident from the observation that a point mutation of the corresponding AP-1 binding sites within the viral upstream regulatory region (URR) of HPV16 or HPV18, almost completely abolishes transcription of URR- driven reporter constructs either under transient transfection assays (Thierry et al. 1992, Butz & Hoppe Seyler 1993) or in stable infection assays in organotypic 'raft' cultures (Parker et al. 1997, Zhao et al. 1997).

In this article, studies have been reviewed to show how an antioxidative agent PDTC can modulate gene activity leading to selective supression of HPV gene expression through redox regulation that changes the composition of AP-1, a transcription factor essential for viral gene expression.

## Human Papillomavirus and Cervical Cancer

Human papillomaviruses are known to cause warts, papillomas, condylomata accuminata and neoplasias of the lower genital tract in humans (zur Hausen 1989, 1996, Das et al. 2000). The viral particles are about 55nm in diameter and contain a double stranded closed circular DNA of about 8000 bp. The DNA is encapsidated by 72 capsomers and replicates as an episome in the host cell nucleus. HPVs belong to papillomaviridae family and is epitheliotropic in nature. Presently, there are nearly 100 different HPV genotypes have been identified but certain specific types of HPVs, such as HPV type 16 and HPV18 which are designated as "high risk" types are predominantly involved in the development of cancer of the uterine cervix in women. HPV types 6 and 11 which cause only benign lesions, warts and papillomas are considered as "low risk" types. The evidence for causal role of HPVs in the development of cervical cancer comes from etiological and epidemiological observations together with the experimental findings of the molecular pathways elicited by HPV transforming genes. Further evidence in favour of papillomavirus as the carcinoma virus comes from the findings of frequent detection of HPV infections in cancers of oral, esophageal, larynx and non-melanoma skin cancers (zur Hausen 1989).

Cancer of the uterine cervix is the second most common cancer in women worldwide (Parkin et al. 2001) but it is the leading cancer in Indian women. In India, the genome of high-risk HPV types 16 and 18 can be detected in almost 100% of cervical carcinomas. Most interestingly, while HPV type 16 alone is detected in more than 90% of carcinomas, the occurrence of other high risk types such as HPV types 31, 33, 35, 39, 45 51, 52, 58, 56, 59 and 68 are very rare or nil in India (Das et al. 1992a). HPV 18 though more aggressive is found only in 3 to 4% of cervical cancers. In malignant tumors, the viral genome is mostly (~70%) integrated into the host cell chromosome while it is always found in an episomal form in benign growths and dysplastic lesions (Das et al. 1992b). During integration, the E1 and E2 genes are often found to be disrupted or deleted. Although the viral genome shows substantial rearrangements or deletions, the E6 and E7 ORFs responsible for tumorigenic phenotype are regularly preserved and remain transcriptionally active in the tumours or in the cell lines derived from them.

The E6 and E7 proteins of high risk HPV type 16 and HPV 18 have been shown to play an important role in tumorigenic transformation of cells but these proteins of low risk HPV types 6 and 11 which are associated with benign lesions normally do not exhibit transforming properties. It has been

demonstrated that the expression of E6 and E7 gene is essential for the maintenance of tumorigenic phenotype of cervical carcinoma cells (Vonknebel et al. 1988, 1992).

### Structural and Functional Organization of HPV

The genome of papillomaviruses consists of a double stranded circular DNA of about 7,900 bp. Figure 1 represents the common genomic organization and functions of HPV ORFs exemplified by HPV 18. Almost half of the genome contains seven open reading frames or early genes designated as E1 to E7 (E for early) as they generally express in basal and suprabasal cells of cervical epithelium. In contrast, the two late genes, L1 and L2, which account for 40% of the genome and encode viral capsid proteins, are expressed in the superficial epithelial layer. The early and late genes are separated by approximately an 800-1000bp (10% of the genome) transcriptional control region called upstream regulatory region or URR. It is also designated as the long control region (LCR) or non- coding region (NCR) since it does not code for proteins. It contains viral promoters and several cis-acting transcriptional enhancer elements, the replication origin and polyadenylation sites. Several viral and host cell transcription factors including AP-1, which

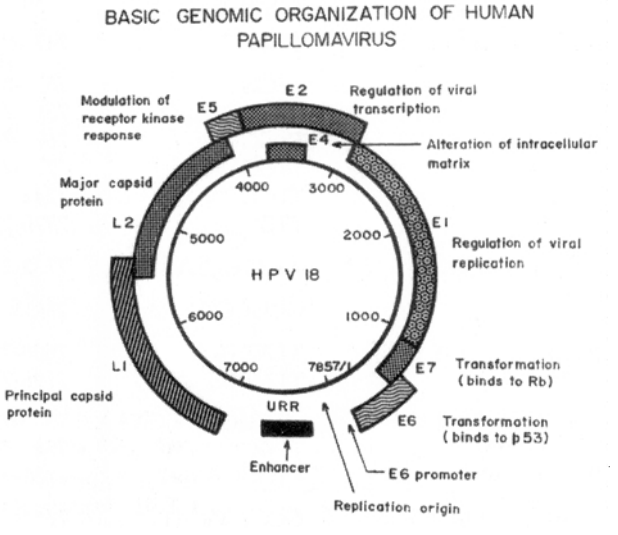


**Figure1** Structural and functional organization of human papillomavirus genome as examplified by HPV type 18.

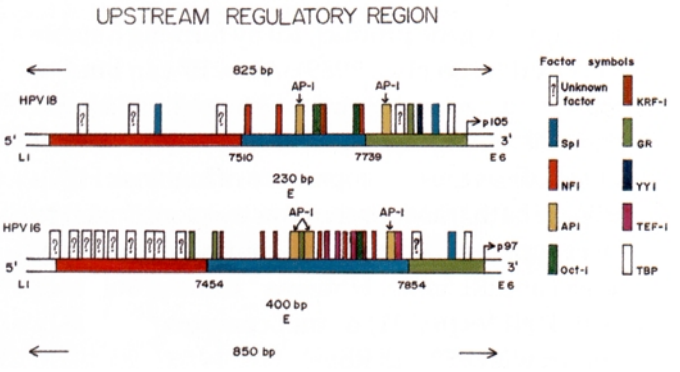


**Figure 2** Schematic diagram of upstream regulatory region (URR) of HPV 18 and HPV 16 showing 5' region, constitutive enhancer and promoter proximal region containing E6/E7 promoters p105 and p97 respectively. The symbols of different transcription factors and their binding sites in URR are indicated.

participate in transcriptional regulation of HPV oncogene expression bind to this URR (see figure 2).

The transcription factors that bind to and activate the transcriptional enhancer elements of HPV 16/18 and all other genital HPVs include activator protein-1 (AP-1), nuclear factor-1 (NF-1), octamer-binding factor-1 (Oct-1) transcriptional enhancer factor 1 and 2 (TEF-1 and TEF-2), the glucocorticoid receptor, the progesterone receptor and the Yin and Yang factor (YY1). (Hope-Seyler & Butz 1994, Mark & Laimins 1991, Bauknecht et al. 1992 (figure 2). Human papillomaviruses are epitheliotropic in nature and the epithelial specificity of its enhancer may be responsible for the tropism of HPV infection and it correlates with particular subsets of NF-1 and AP-1 transcription factors. The other biological important functions of the enhancer include stimulation of HPV gene expression by progesterone during pregnancy or menstrual cycle and influence certain anti-ovulants leading to progression. An extremely long persistence of HPV infections and a slow progression of lesions indicate that replication as well as transcription are carefully modulated by the virus.

As regards functions of different ORFs, studies have revealed that the E6 and E7 of high risk HPVs are the principal transforming genes (Munger et al. 1989). The E6 protein of HPV 16/18, similar to adenovirus E1B and SV40 large T can form an inactivating complex with p53 tumor suppressor gene product which gets degraded through ubiquitin pathways (Werness et al. 1990) while the E7 protein inactivates the retinoblastoma

susceptibility gene product, Rb by forming a stable complex (Dyson et al. 1989). Also, E7 can bind to cyclins and cyclin dependent kinases (cdks) and help in disrupting regulation of cell cycle (Soppler et al. 1994). Thus these oncoproteins of high risk HPVs abrogate both transcriptional activation as well as cancer suppression or growth control properties of the p53 and Rb gene. However, these proteins of low risk HPVs (6 / 11) cannot complex or weakly complex with p53 and Rb.

As of other ORFs, E1 is the most conserved among different HPV types and is responsible for HPV replication as well as for the site-specific DNA binding activity. E2 faciliates E1 binding to the replication origin located proximal to the URR and codes for at least three proteins which act as transcription factors (Boward et al. 1994). In high risk HPV types 16/18, E2 binds to URR and acts as a transcriptional activator. The E2 is mostly deleted or disrupted in cervical tumors and cell lines derived from them. This seems to facilitate integration of HPV into the host cell genome (Cripe et al. 1987, Das et al. 1992b). This is supported by the observation that mutation in E2 ORF or its binding sites within the URR leads to an enhanced immortalization activity of HPV 16. The loss of E2 leads to derepression of the E6/E7 promoter and overexpression of E6 and E7 proteins leading to malignant transformation. The ORF coding for E5 protein also shows transforming activity and stimulates mitogenesis (Stoppler et al. 1994) but mostly in bovine papillomavirus (BPV) and is frequently deleted in cervical cancer (Schwarz et al. 1985). The role of E4 ORF is not very clear but it is suspected to have a role in productive infection of the virus and distribution of the cytokeratin network. The E3 ORF is known to present in HPVs but its function is not known. Of two late gene proteins (L1 and L2), the L1, a 55 kDa protein represents about 80% of total capsid protein and constitutes the basic structure of viral capsid. The L2, 70kDa protein constitutes the remaining 20% of capsid and possibly helps in viral assembly (Howley 1996).

## Upstream Regulatory Region (URR)

The Upstream regulatory region (URR) plays a central regulatory role in HPV-associated cell transformation. The promoter for E6/E7 transcription is located at the 3′ end of the URR. The HPV 16 URR constitutes a 850 bp region which contains (i) a 5′ terminal portion of unknown function, (ii) a central constitutive enhancer of 400 bp and (iii) a promoter proximal region containing the E6/E7 promoter p97 at the 3′ end. The 825 bp URR of HPV 18 is also divided into the above three regions and its promoter is located at p105.(See figure 2; also see Thiery et al. 1987, Cripe et al. 1987, Hoppe-Seyler & Butz 1994). These URRs along with their enhancers have been shown to be epitheliotropic in their transcriptional activity. Different transcription factors binding motifs within the URRs of HPV 16 and HPV 18 are shown in table 1.

## Viral Replication and Transcription

The pathological effect of HPV is primarily confined to epithelium and there exists a close association between viral replication and squamous epithelium differentiation. When the virus enters the basal stem cells of epithelium the viral early genes are amplified and forms the DNA plasmids. Expression of early

**Table 1** *Host-Cell Transcription factor binding motifs within the URRs of HPV 16 and HPV 18.*

| Virus type | Binding site (Transcription Factor) | Sequences 5′→3′ | Nucleotide Position |
|---|---|---|---|
| HPV 16 | AP-1 | TTAATCA | 7631-7637 |
| | | TGAGTCA | 7648-7654 |
| | | TTGTCA | 7810-7816 |
| | NF-1 | TTGGC | 7473-7477 |
| | | TTGGC | 7558-7554 |
| | | TTGGC | 7591-7587 |
| | | TTGGC | 7679-7675 |
| | | TTGGC | 7711-7715 |
| | | TTGGC | 7742-7746 |
| | | TTGGC | 7770-7766 |
| | Oct-1 | AATTGCAT | 7732-7739 |
| | Sp-1 | GGGCGT | 28-33 |
| HPV 18 | AP-1 | TTAGTCA | 7608-7614 |
| | | TTAGTCA | 7798-7614 |
| | NF-1 | CTGGCN$_5$TGCAAA | 7513-7528 |
| | | TTGGCN$_8$TTGGC | 7569-7586 |
| | | TTGGC | 7731-7735 |
| | Oct-1 | GCCTTGCAT | 7644-765 |
| | | AATTGCAT | 7721-772 |
| | Sp-1 | GGGAGT | 35-40 |

genes also stimulates cell growth and differentiation in basal layer and later viral replication and late gene expression are activated in the upper layer of the fully differentiated epithelial cells leading to the production of infectious viral particles (Howley 1996, Chow & Broker 1994). Transcription of HPV is controlled by several factors such as multiple promoters, multiple splice patterns, different polyadenylation signals and several proteins and factors produced by the virus as well as the host.

**Role of Transcription Factors in Gene Expression**

The basic biological phenomenon that helps in building and maintaining an organism is the regulation of gene expression. It comes to play a role in situation as wide ranging as maturation of antibody secretary cells to transformation of a fertilized ovum into an embryo. Almost all diseases that clinicians encounter daily in their clinics are examples of normal or abnormal expression of genes. For example, most cancers arise due to abnormalities in the expression of genes concerned with cellular growth and differentiation. Metabolic disorders or altered hormone actions are also the results of abnormal gene expression. The expression of these genes is regulated by a class of cellular proteins called transcription factors which bind to regulatory elements such as promoters and enhancers in the DNA. They stimulate or sometimes initiate gene transcription and thereby leading to formation of messenger RNAs through direct interaction with DNA. Transcription factors are therefore essential for normal cellular growth, development and differentiation and for a large number of cellular responses to external stimuli.

Extensive studies on many eukaryotic genes which are regulated in an inducible, cell type-specific or constitutive fashion have revealed three basic facts that (I) specific short sequences, the cis-acting elements which are located near to or within genes regulate gene expression, (ii) these sequence elements tend to bind sequence-specific DNA binding proteins, called the trans-acting factors and (iii) binding of proteins to DNA is responsible for the initiation, maintenance and up or down regulation of transcription. The cis-acting elements controlling gene expression and transcribed by RNA polymerase II are called promoters, enhancers or silencers. Promoters are found to a position neighbouring the start site of transcription and function in an orientation-dependent fashion. But enhancers/silencers generally modulate the activity of promoters and are flexible with regard to their orientation and position from the transcription start site. All three types of cis-acting elements have the binding sites for multiple distinct DNA binding proteins. Protein binding DNA sequences in promoters, enhancers/silencers can differ in their affinity and specificity for a given protein. One of the well-studied transcription factors responsible for inducible gene expression is activator protein-1, AP-1. It indicates that induction of DNA-binding is a common mechanism to turn on transcriptional activator. AP-1 increased DNA binding by heterodimer formation between its dimerization partners, jun and fos proteins.

AP-1 is formed by dimerization of jun and fos proteins via hydrophobic interactions of 'Leucine-Zippers' (Bohmann et al. 1987, Angel et al. 1988, Chiu et al. 1988). Recently, other members of the jun family i.e., JunB and Jun D and of the fos i.e., fosB1, fosB2, fra-1 and fra-2 have been identified. AP-1 plays a pivotal role in controlling expression of genes involved in cellular proliferation, differentiation and neoplastic transformation (Angel & Karin 1991). AP-1 has also been found to play a central role in the expression of human papillomavirus genes. It was first identified as a transcription factor that binds to an essential cis-element of the human metallothionein IIa (hMTIIa) promoter (Lee et al. 1987). Binding of AP-1 is necessary for transcriptional activation of AP-1 dependent genes following PKC activation (Karin & Herrlich 1989, Karin 1989).

**Role of Redox State in Gene Expression**

The activity of certain transcription factors is known to be modulated by redox status of the cells. This is achieved by inducing prooxidant states within the cell by generating reactive oxygen intermediates (ROIs) (Goosens et al. 1995, Mayer et al. 1993). Following binding to their receptors on the cell surface, intracellular ROIs transduce the signal into the nucleus by acting as second messenger (Schreck et al. 1991). Reactive oxygen species (ROS) are strongly implicated in the

pathogenesis of wide variety of human diseases. To understand ROS-dependent disorders in biological systems, the regulation of gene expression by oxidants and antioxidants or other determinants of the intracellular reduction-oxidation (redox) state have been investigated. At least, two well-defined transcription factors, AP-1 and NFkB have been shown to be regulated by the intracellular redox status. The binding sites of AP-1 and NFkB are located in the promoter region of large variety of genes that are directly involved in the pathogenesis of diseases such as cancer, AIDS, diabetes, atheosclerosis etc. Biochemical and clinical studies have therefore suggested antioxidant therapy for the treatment of these diseases. Critical steps in the signal transduction pathways are sensitive to antioxidants and several cell regulatory events such as phosphorylation, binding of transcription factors to consensus site on DNA are dependent on the intracellular oxidant-antioxidant homeostasis. Therefore, the antioxidative agents are considered to be effective regulators of redox-sensitive gene expression.

Considering that there may involve a redox-regulatory pathways in the expression of HPV oncogenes which needs involvement of AP-1, the transcriptional pattern of HPV has been studied using a redox model. For this a synthetic antioxiadative agent, pyrrolidine dithiocarbamate (PDTC) which is a potent oxygen radical scavenger, has been used (Rösl et al. 1997).

The cell lines used for the experiments were HPV 16 immortalized human keratinocytes HPK-1a, HPV 18-positive HeLa and HPV-negative carcinoma cell line C33a. Treatment of these cells with the antioxidant in micromolar concentration can completely abolish the viral gene (E6 and E7) expression between 4 to 6 hours of treatment (figure 3). It was found that the antioxidant - induced time-dependent down regulation of HPV transcription is a selective process and not the consequence of general transcriptional reduction since normal cellular genes such as β-actin and c-myc remain largely unaffected under same experimental conditions. Although 2 to 3 fold reduction of the short-lived c-myc mRNA can be noticed (figure 3), the basic steady state level of

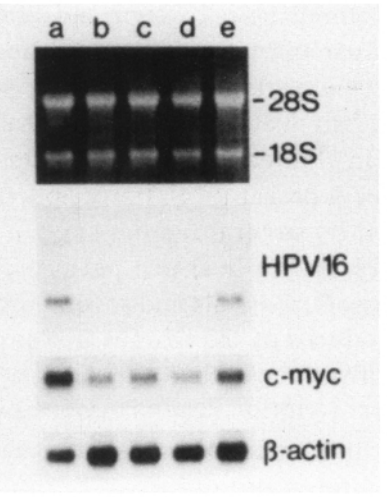


**Figure 3** Northern blot analysis showing down regulation of HPV 16 immortalized human keratinocytes (HPK 1a) treated with PDTC for different periods. The position of 28S and 18S rRNA are indicated in ethidium bromide-stained agarose gel. Lane a, untreated control, lanes b to e, overnight, 6h, 4h and 2h of incubation in the presence of PDTC respectively. (Ref: Rösl et al. 1997)

transcript remains constant and is almost unaltered independent of incubation for 4hr. or 16 hr. In experiments with a non-specific transcription inhibitor, actinomycin D, it was also established that PDTC acts at the initiation of transcription as HPV transcripts declined between 120 to 240 min., which is also estimated to be the half-life of mRNA (Rösl et al. 1997).

Although p53 is known to down regulate HPV transcription and can also be redox - regulated but PDTC does not affect p53 or p53 responsive gene, WAF-1/P-21. Most interestingly, PDTC can induce elevated binding of AP-1 to its cognate recognition sites within the viral regulatory region (URR). This is intriguing since AP-1 is indispensable for HPV expression but the expression of HPV transforming genes E6/E7 is suppressed (figure 4). It is also observed that PDTC treatment selectively increased AP-1 binding but other transcription factors are not affected (see figure 4). Elevated AP-1 binding was observed in HPK1, HeLa including C33a cervical cancer cells lacking HPV. It suggests that PDTC-induced increased AP-1 binding is a general phenomenon and is not exceptional for HPV positive cells. To understand how elevated binding of AP-1 could be involved in the

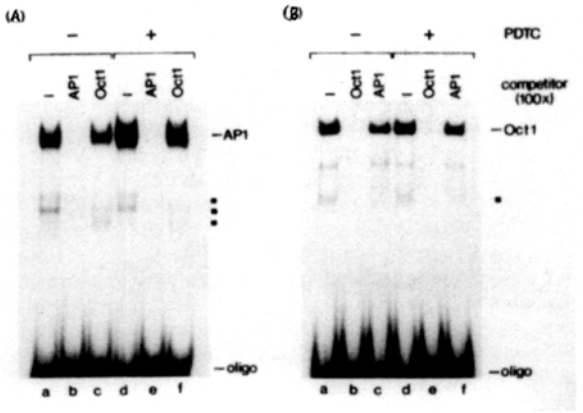


**Figure 4** (A, B) Electrophoretic mobility shift assay (EMSA) using cellular extracts from untreated (-) and PDTC treated (+) HPK1a cells – Binding specificity is evidenced by incubation with a 100 fold molar excess of the homologous unlabelled oligonucleotide (lanes b and e in each panel) in comparison with competition experiments using a heterologous oligonucleotide (lanes c and f in each panel).
(A) EMSA with $^{32}$p-labelled oligonucleotide harbouring an AP-1 consensus sequence,
(B) EMSA using a labeled oligo encompassing a consensus sequence of the Oct-1 transcription factor. The squares indicated the unspecific complexes. (Rosl et al. 1997)

suppression of HPV expression, AP-1 complex was dissected out in their individual components in electrophoretic mobility band supershift assays using specific antibodies raised against different members of jun and fos family. Also, analysis of all component of AP-1 at RNA and protein level revealed that the normal composition of AP-1 consisting of jun homodimer has been altered. Jun protein was found to be phosphorylated and heterodimerized not with its normal partner c-fos inspite of its high rate of synthesis but primarily with a fos- related antigen Fra-1 (figure 5) which can be induced by PDTC or tumor necrosis factor α (TNF α). Northern blot analysis of RNA extracted from HPV 16 positive HPK 1 and HPV 18 positive HeLa cells following treatment of PDTC and after hybridization with c-jun, c-fos and fra-1 specific cDNA probes indicates that PDTC do induce c-Jun and Fra-1- expression. Since elevated level of Fra-1 is known to abrogate transactivation of AP-1 prototype c-jun/c-fos complexes on AP-1 responsive genes (Suzuki et al. 1991, Yoshioka et al. 1995), it is possible that

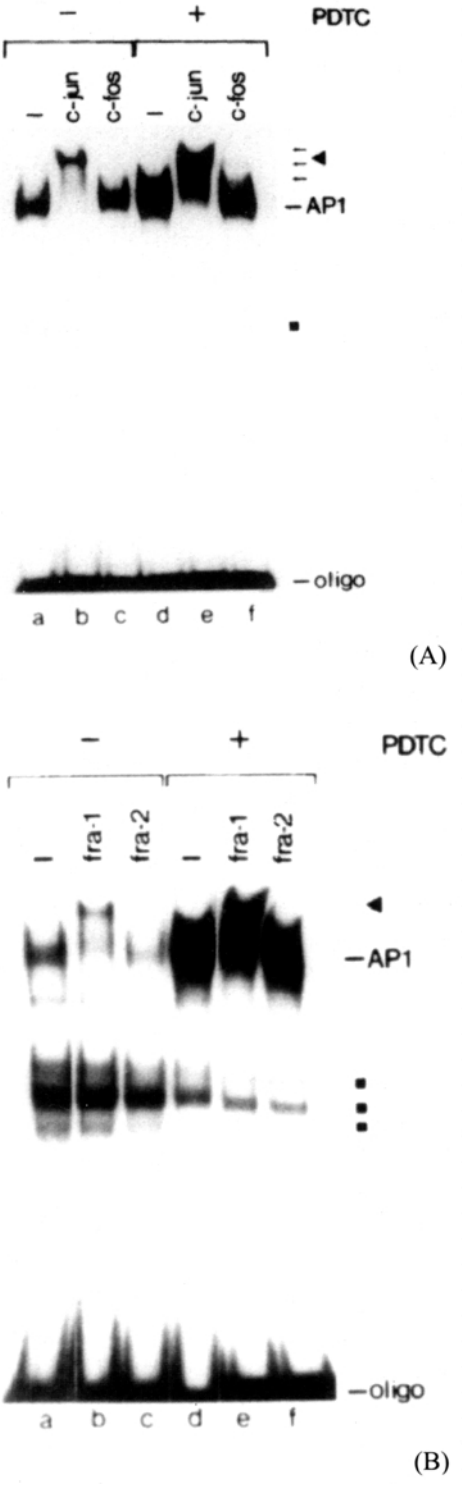


**Figure 5(A, B)** Electrophoretic mobility shift assay using $^{32}$P-labelled consensus AP-1 nucleotides in extracts from untreated (-) and PDTC treated (+) HPK1a cells incubated with specific antibodies against c-jun, c-fos (A) and fra-1 and fra-2(B). Selective induction of c-jun and no effect on c-fos is shown (A) after PDTC treatment, while (B) shows selective induction of fra-1 in supershifts.

suppression of HPV oncogene expression is mainly due to reorganization of AP-1 transcription complex following PDTC treatment.

### The Role of c-Jun, c-Fos and Fra-1

To determine the level of gene expression and the extent of involvement of jun and fos members, particularly the fra-1 within the AP-1 complex during cervical carcinogenesis, individual members were analysed in an interesting experimental model. Cell lines used were derived from somatic cell hybrids between tumorigenic HeLa cells and normal human fibroblasts (Stanbridge 1982). Three cell lines e.g., HeLa cells, its highly tumorigenic segregant, CGL3 and non-tumorigenic segregant, 444 cells-all positive for HPV 18 (Stanbridge 1982) were used for the study.

It has been observed that in nontumorigenic 444 cells, c-jun represented the major dimerization partner followed by jun D and jun B. but in tumerigenic cells of HeLa or CGL3, there is less of c-jun and more of jun B. On the other hand, dissection of individual members of fos-family within AP-1 complex revealed Fra-1 as the major dimerization partner of c-jun and jun D in 444 cells. This is in marked contrast to HeLa and CGL3 cells where Fra-1 is either extremely low or completely absent but c-fos is detectable in much higher level while 444 cells completely lacked c-fos expression. Fos B is not traceable in all cell lines. Thus Fra-1 has been found to be a major dimerization partner in the functioning of AP-1 which is indispensable for initiation and maintenance of HPV transcription. Interestingly, tumor necrosis factor alpha (TNF α) which selectively suppresses HPV 18 transcription in 444 cells, can induce Fra-1 binding which seems to increase as a function of duration of cytokine treatment in 444 cells, but very low in HeLa and nil in CGL3. (figure 6).

It is interesting to note that both HeLa and CGL3 cells lack the normal allele of chromosome 11. The role of fra-1 in the context of HPV negative regulation could be of functional importance since the fra-1 is located in chromosome 11q13 region (Sinke et al. 1993) which is suspected to harbour tumour supressor gene(s) and is often structurally

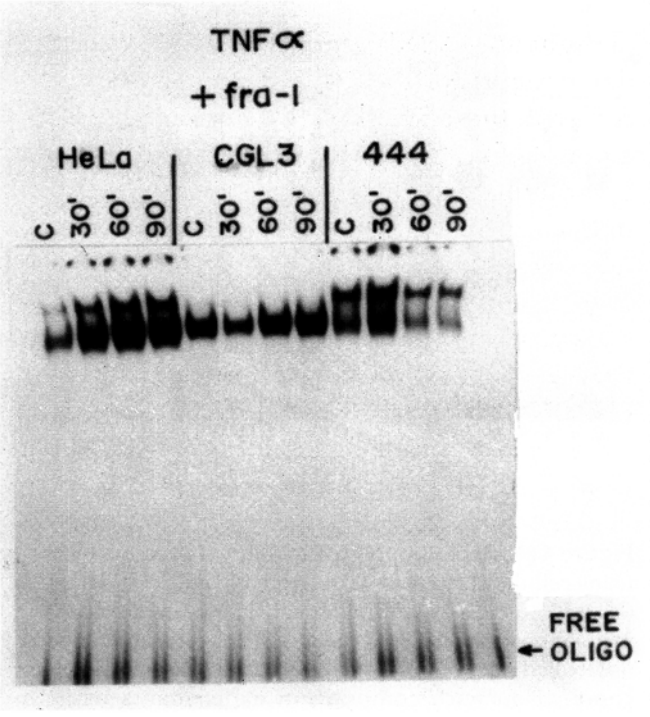


**Figure 6** Determination of time-dependent Fra-1 induction in electrophoretic mobility supershift assays (EMSAs) in untreated control (c) and TNF α treatment for different periods in HPV 18 positive non-tumorigenic hybrids 444 cells, tumorigenic HeLa and highly tumorigenic CGL3 cells. Supershift EMSA after treatment of TNFα for 30, 60 and 90 min and Fra-1 antibody addition. Note complete absence of Fra-1 supershifts in CGL3.

deleted or rearranged in cervical cancer cells (Jesudasan et al. 1995). It is also shown that enhanced fra-1 expression can abolish transactivation of c-jun/c-fos complexes on AP-1 responsive genes. However, the overexpression of Fra-1 alone failed to cause significant supression of HPV expression. Its participation as a major dimerization partner within the AP-1 complex including its location at a chromosome 11 known to have tumor suppressor activity – neverthless points to a negative regulatory or possible tumor suppressor activity of fra-1 in the multistep process of cervical carcinogenesis. It is not unlikely that certain other protein(s) or yet unknown upstream or downstream signaling pathways may be involved.

### Ectopic Expression of c-Fos in Nontumorigenic Cells and Induction of Tumorigenicity

Fra-1 is induced at transcriptional as well as at protein level by TNF α exclusively in non-

malignant 444 cells which correlates with the down regulation of HPV 18 gene expression. In contrast, tumorigenic cells harbour very high amounts of c-fos while non-tumorigenic cells completely lack c-fos (Soto et al. 1999). It indicates that c-fos plays a crucial role during tumorigenic transformation of cervical epithelial cells. In order to test whether overexpression of c-fos in nontumorigenic cells could complete out Fra-1 expression from AP-1 complex and induce tumorigenicity, transfection experiments were carried out with c-fos gene. An expression vector 'pMSc-fos' under the control of SV40 promoter/enhancer encoding a cDNA of c-fos gene was stably transfected into nontumorigenic 444 cells. Different G418 (neomycin) resistant clones harboring an intact transcription cassette were selected. Clones which did not contain exogenous c-fos gene were used as negative controls. In order to test whether these clones differ in their in vivo growth properties, different clones were subcutaneously inoculated into two flanks of 6 weeks old athymic nu/nu mice. After 6 months, none of these clones except clone 1 which expressed high level of c-fos developed tumors. The clones which failed to develop tumors were tested to have a low level of c-fos expression. Re-examination of tumor specimens obtained from both flanks of tumor-bearing mice confirmed that c-fos is highly expressed but Fra-1 is completely down regulated or absent. The tumor formation was accompanied by a counter selection against Fra-1 expression. C-jun, Jun-B, Jun-D as well as other fos family members such as Fra-2 and Fos-B are neither modified nor detected in tumorigenic clones (Soto et al. 1999).

The modifications of initial AP-1 complex towards AP-1 profile specific of tumorigenic CGL3 and HeLa cells may raise the question whether cells obtained from explanted tumors are still sensitive to cytokines. To answer this, expression of HPV 18 E6/E7 was monitored in northern blots after challenging with TNF α for different periods. Interestingly, HeLa or CGL3 cells and none of the reactivated clones showed any effect of TNF α on viral gene expression. This is in sharp contrast to what have been observed in parental 444 cells. All these observations emphasize an important role of AP-1 in HPV-mediated cell transformation. The observation that overexpression of c-fos rendered HPV 18 transcription refractory to TNF α indicates a causal link between cytokine sensitivity and AP-1 composition in HPV negative regulation.

## Conclusions

Transcription factor AP-1 is found to be an indispensable key regulator of epithelial tissue-specific transcriptional activity of various HPV types. In cervical cancer, the composition of transcription factor AP-1 plays not only an important role as a positive regulator of HPV transcription but is also considered as a central controlling element within the intracellular surveillance mechanism negatively regulating HPV directed E6/E7 expression during progression of non-malignant cervical cells to malignant cells. An increased expression of c-fos within the AP-1 complex plays a crucial role during tumorigenic transformation. And, the antioxidative agents such as PDTC can bring about modification in this important transcription factor to interfere with the regulation of HPV transcription resulting in selective suppression of viral gene expression. These results provide a molecular basis for development of possible novel chemotherapeutic approaches to control pathogenic HPVs by designing anti-oxidative drugs or antioxidant gene therapy. It is suggested that antioxidants with antineoplastic / antiviral properties from natural sources will be most promising targets for chemotherapeutic drug development.

It would be therefore most interesting to screen our rich source of natural / harbal antioxidants which are in human use in various forms for ages and are easily acceptable to human body for their capacity to down regulate viral or other oncogene expression by acting on transcription factor(s) that are important targets for therapeutic interventions.

## References


Angel P and Karin M 1991 The role of jun, fos and the AP-1 complex in cell proliferation and transformation; *Biochem Biophys Acta* **1072** 129-157

Bauknecht T, Angel P, Royer H and zur Hausen H 1992 Identification of a negative regulatory domain in the human papillomavirus type 18 promoter interaction with the transcriptional repressor YY1; *EMBO J* 11 4607-4617

Bouvard V, Storey A, Pim D and Banks L 1994 Characterization of the human papillomavirus E2 protein: evidence of trans-activation and trans-repression in cervical keratinocytes; *EMBO J* 13 5481-5459

Butz K and Hoppe-Seyler F 1993 Transcriptional control human papillomavirus (HPV) oncogene expression: composition of the HPV type 18 upstream regulatory region; *J Virol.* 67 6476-6486

Butz K and Hoppe-Seyler F 1993 Transcriptional control of human papillomavirus (HPV) oncogene expression: composition of the HPV type 18 upstream regulatory region; *J. Virol.* 67 6476-6486

Chow L T and Broker T R 1994 Paillomavirus DNA replication; *Intervirology* 37 150-158

Cripe T P, Haugen T H and Turk O P et al. 1987 Transcriptional regulation of the human papillomavirus – 16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 transactivator and repressor gene products: Implications for cervical carcinogenesis; *EMBO J* 6 3745-3753

Das B C, Sharma J K Gopalkrishna V, Das D K, Singh V, Gissmann L, zur Hausen H and Luthra U K 1992a A high frequency of human papillomavirus DNA sequences in cervical carcinomas of Indian women as revealed by Southern blot hybridization and polymerase chain reaction; *J. Med. Virol.* 36 239-245

Das B C, Gopalkrishna V, Hedau S and Katiyar S 2000 Cancer of the uterine cervix and human papillomavirus infection; *Current Science* 78 52-63

Das B C, Sharma J K, Gopalkrishna V and Luthra U K 1992b Analysis by polymerase chain reaction of the physical state of human papillomavirus type 16 DNA in cervical preneoplastic and neoplastic lesions; *J. Gen. Virol.* 73 2327-2336

Dyson N, Howley P M, Munger K and Harlow E 1989 The human papillomavirus-16 E 7 oncoprotein is able to bind to the retinoblastoma gene product; *Science* 243 934-936

Goossens V, Grooten J, deVos K and Fiers W 1995 Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity; *Proc. Natl Acad Sci USA* 92 8115-8119

Hoppe-Seyler F and Butz K 1994 Cellular control of Human papillomavirus oncogene transcription; *Molec. Carcinogenesis* **10** 134-141

Howley P M 1996 Papillomaviridae and their replication; in *Virology*, 3rd Edn, Eds B N Fields et al. Chapter 65, pp2045-2076 (Philadelphia: Lippincoit-Raven)

Hunter T and Karin M 1992 The regulation of transcription by phosphorylation; *Cell* 70 375-387

Jesudasan R A, Rahman R A, Chandrashekharappa S Evans G A and Srivastan E 1995 Deletion and translocation of chromosome 11q13 sequences in cervical carcinoma cell lines; *Am. J. Hum. Genet.* 56 705-715

Lee W, Haslinger A, Karin M and Tjian R 1987 Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40; *Nature* 325 368-372

Munger K, Phelps W C, Bubb V, Howley P M and Schlegel R 1989 The E6 and E7 genes of the human papillomavirus type 16 are necessary and sufficient for transformation of primary human keratinocytes; *J. Virol.* 63 4417-4421

Parker J N, Zhao W, Askins K J, Broker T R and Chow L T 1997 Mutational analysis of differentiation-dependent human papillomavirus type 18 enhancer elements in epithelial raft culture of neonatal foreskin keratinocytes; *Cell Growth Differ* 8 751-762

Parkin D M, Bray F, Ferlay J and Pisani P 2001 Estimating the world cancer burden: Globoscan 2000; *Int. J. Cancer* 94 153-156

Rösl F, Das B C, Legert M, Geletneky K and Zur Hausen H 1997 Antioxidant-induced changes of the AP-1 transcription complex are paralleled by a selective suppression of human papillomavirus transcription; *J. Virol* 71 362-370

Schreck R, Rieber P and Baeuerle P A 1990 Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kB transcriptions factor and HIV-1; *EMBO J* 10 2247-2258

Schwaz E, Preese U K, Gissmann L et al. 1985 Structure and transcription of human papillomavirus sequences in cervical carcinoma cells; *Nature* 31 111-114

Sinke R J, Tanigami A, Nakamura Y and Geruts van Kessel A 1993 Reverse mapping of the gene encoding the human fos related antigen-1 (fra-1) within chromosome band 11q13; *Genomics* 18 165

Soto U, Das B C, Lengert M, Finzer P zur Hausen H and Rosl F 1999 Conversion of HPV 18 positive non-tumorigenic HeLa-fibroblast hybrids to invasive growth involves loss of TNF-a mediated repression of viral transcription and modification of the AP-1 transcription complex; *Oncogene* 18 3187-3198

Stanbridge E J, Der C J, Doersen C J, Nishimi R Y, Peehl D M, Weissman B E and Wilkinson J E 1982 Human cell hybrids: analysis of transformation and tumorigenicity; *Science* 215 251-259

Stoppler H et al. 1994 Transforming proteins of the papillomaviruses, Intervirology 37 168-179.

Suzuki T, Okuno H, Yoshida T, Endo T, Nishina H and Iba H 1991 Difference in transcriptional regulatory function between c-fos and fra-2; Nucleic Acid Res 19 5537-5542

Thierry F, Spyrou G, Yaniv M and Howley P M 1992 Two AP-1 sites binding jun B are essential for human papillomavirus type 18 transcription in keratinocytes; *J. Virol.* 66 3740-3748

Thiery F Heard J M Darmann K and Yaniv M 1987 Characterisation of a transcriptional promoter of human papillomavirus 18 and modulation of its expression by simian virus 40 and adenovirus early antigens; *J. Virol.* 61 665-672

Von Knebel, Doeberitz M, Otterodorf T and Gissmann L 1988 Correlation of modified human papillomavirus early genes expression with altered growth properties in C4-1 cervical carcinoma cells; *Cancer. Res* 48 3780-3786

Von Knebel, Doeberitz M, Rittmiller C, zur Hausen H and Durst M 1992 Inhibition of tumorigenicity of C4-1 cervical cancer cells in nude mice by HPV 18 E6-E7 antisense RNA; *Int. J. Cancer* 51 831-834

Werness B A, Levine A J and Howley P M 1990 Association of human papillomavirus type 16 and 18 E6 proteins with p53; *Science* 248 76-79

Yoshioka K, Deng T, Cavigelli M and Karin M 1995 Antitumor promotion by phenolic antioxidants: inhibition of AP_1 activity through induction of Fra-1 expression; *Proc. Natl. Acad. Sci. USA* 92 4972-4976

Zhao W, Chow L T and Broker T R 1997 Transcription activation of HPV type 11 E6 promoter-proximal elements in raft and submerged cultures of foreskin keratinocytes; *J. Virol.* 71 8832-8840

Zur Hausen H 1989 Papillomavirus as carcinoma viruses; pp 1-26 eds G Klein (New York: Raven Press)

_____ 1996 Human papillomavirus-a major cause of human cancers; *Biochem. Biophys Acta* 1288 55-78