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Human lung, bladder and head and neck tumors as compared to their adjacent normal tissues have elevated AP-1 activity and recognize sequence elements of HIV-1 LTR

V. ZOUMPOURLIS[1,2], E. PAPADAKIS[1,3], D. DELAKAS[2], A. CRANIDIS[2], J. SEGAS[2], H. PAPADAKIS[2] and D.A. SPANDIDOS[1,2]

[1]Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, Athens; [2]Medical School, University of Crete, Heraklion; [3]Metaxa Anticancer Hospital, Pireas, Greece

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Abstract. We have previously reported the specific binding of nuclear factor AP-1 isolated from human breast MDA MB 468 and HeLa cervical tumor cell lines to oligonucleotides complementary to three newly elucidated sequences within the HIV-1 LTR. These synthesized oligonucleotides, which bear high homology to the AP-1 recognition sequence, were used in the present study in gel retardation assays together with unfractionated nuclear protein extracts from human lung, bladder and head and neck tumors and adjacent normal tissue to study the role of the AP-1 protein in the regulation of HIV-1 expression. We found increased binding of AP-1 to these oligonucleotides in 9/12 lung tumors, 9/14 bladder tumors and 7/7 head and neck tumors as compared to adjacent normal tissues. This confirms previous results obtained when using MDA MB 468 and HeLa nuclear protein extracts. These results indicate that, AP-1 could be contributing to the HIV-1 transcriptional regulation through its interaction with the AP-1 binding sites of HIV-1 LTR.

Introduction

It is well established that the human immunodeficiency virus type 1 (HIV-1) is the main etiologic agent of acquired immunodeficiency syndrome (AIDS), which destroys the immune surveillance system and thus causes various pathological symptoms to develop (1-3). The typical long latency before onset of symptoms may be related to various stimuli or cofactors, which are involved in the disruption of the latent stage of HIV and might lead to provirus transcriptional activation. Human and viral proteins (4-6) and the oncoprotein *ras* p21 (7) may contribute to the transition of HIV-1 from the latent stage to the infectious state. Different biological response modifiers (insulin, epidermal growth factor, tumor necrosis factor) (8,9), hormones (hydrocortisone, dexamethasone) (8) and antitumor drugs (*cis*-platin, doxorubicin, daunomycin, hexamethylene bisacetamide and mitomycin C as opposed to carboplatin) (10-16), increase the chloramphenicol acetyl transferase expression from the HIV-1 LTR sequence in human and rat fibroblasts.

The human immunodeficiency virus type 1 long terminal repeat, HIV-1 LTR, contains binding sites for several cellular transcription factors which are implicated in HIV-1 gene expression. The regulatory sequences present upstream of the transcription initiation site (+1) can be broadly categorized into proximal (promoter) and distal (enhancer) elements. The promoter element contains the TATA box, located between nucleotides -22 to -27, which is presumably recognized by the transcription factor TFIID (17). The HIV-1 promoter also contains three GC-rich sequences, which bind to the ubiquitous transcription factor Sp1 (17,18). In addition to the upstream regulatory sequences, the HIV promoter contains several protein-binding sites downstream of the RNA start site. A leader binding protein (LBP-1) binds to a region from -17 to +27 and TF/NF-1 binds to a sequence from +40 to +45 (19). Furthermore, the untranslated region of the binding protein (UBP-1) has been shown to recognize HIV-1 LTR sequences from -13 to +28 of the nucleotide (20).

The HIV-1 LTR enhancer (-103 to -81) is recognized by EBP-1 in resting T cells (20) and NF-κB (21) and HIVEN (22) in activated T-cells. *cis* elements include the negative regulatory element (NRE), located in the region between nucleotide positions -357 and -185 relative to the transcription initiation site +1. The NRE contains a number of recognition sequences for cellular transcription factors such as USF, URS and NFAT-1 (6,23), as well as two adjacent binding sites for the transcription factor AP-1 which are located at nucleotides -348 to -343 and -336 to 331 (24).

The AP-1 is a family of related transcription factors encoded by the members of the *jun* and *fos* multigene families that have similar binding characteristics and sequences (25-30). The AP-1 DNA-complex is composed of homo- and heterodimers of the c-*fos* and c-*jun* family (31)

Correspondence to: Professor D.A. Spandidos, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Avenue, 116 35 Athens, Greece

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which dimerize by interdigitation of hydrophobic helices, termed leucine-zippers (32). AP-1 can serve as both a positive and a negative regulator with respect to the expression of a variety of genes under different conditions.

The consensus DNA recognition site for AP-1 is TGAGTCA (33). Two binding sites for transcription factor AP-1 have been mapped within the NRE of HIV-1 LTR (24). In our previous study we examined the HIV-1 LTR and identified three additional AP-1 binding sites (-516 to -495, 310 to -287 and +16 to +42) one of which was found within the TAR element. In the present study binding of AP-1 to these sites has been successfully tested in extracts from lung, bladder and head and neck tumors. The AP-1 activity is elevated in 9/12 lung tumors, in 9/14 bladder tumors and in 7/7 head and neck carcinoma tumors as compared to normal human tissue. These results are in good agreement with our previous study (34) and suggest that these new HIV-1 LTR putative AP-1 sites have the same binding effect as nuclear extracts from HeLa and MDA MB 468 tumor cell lines.

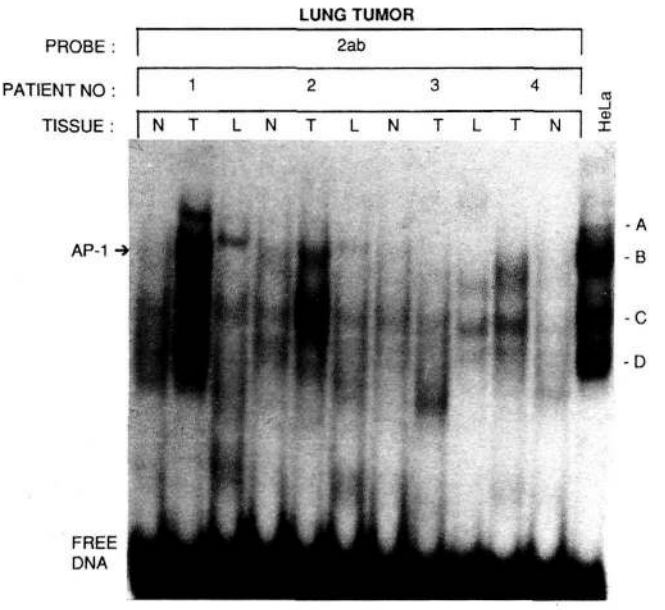

Figure 1. Electrophoretic mobility shift analysis using 2ab-AP-1 binding consensus oligonucleotide. 2000 cpm γ^{32}P-oligo were mixed with 20 μg of nuclear extracts from lung tumors, lymph nodes and adjacent normal tissues and HeLa cells. The DNA-AP-1 protein complex is indicated by the arrow. N = normal, T = tumor, L = lymph nodes.

Materials and methods

Preparation of cell extracts. All biopsy material was obtained from patients who had undergone lung surgery at Metaxa Hospital, Pireas, Greece or bladder and head and neck surgery at the University Hospital, Heraklion, Greece. The tissue was cut into small pieces and homogenized in 2 ml hypotonic buffer (25 mM Tris-HCl pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF) at 5-10 mg protein/ml. The nuclei were pelleted at 2500 rpm in a Sorvall SS34 rotor for 10 min at 4°C. The pellets were washed 3 times with 2 ml isotonic buffer (25 mM Tris-Cl pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.1 M sucrose, 0.5 mM DTT, 1 mM PMSF), resuspended in nuclei extraction buffer (25 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.1% NP40, 0.5 mM DTT, 0.5 mM PMSF) and were further clarified after centrifugation at 25000 rpm in a Beckman Ti 50 rotor for 60 min at 4°C. The supernatant was removed and the extracts were stored at -70°C. Protein estimation was performed as described by Bradford (35).

Preparation of double stranded oligonucleotides and retardation assays. DNA oligonucleotides were as described (34). The oligonucleotides were purified as described (34) and the double stranded oligonucleotides were 5' end-labelled using γ^{32}P-ATP and T4 polynucleotide kinase and end filled using the Klenow fragment of DNA polymerase according to Maniatis *et al* (36).

Results

Four short DNA regions which are putative AP-1 binding sites were identified in the HIV-1 LTR (34). From these four sequences, region 2 shows the highest homology (85.8%) to the AP-1 consensus (5'-TGAGTCA-3') and therefore the corresponding oligonucleotide hybrid 2ab was used in our previous studies to test the affinity of the AP-1 protein in MDA MB 468 and HeLa tumor cells. In the present study the same oligonucleotide sequence was used to examine the presence of AP-1 activity in lung, bladder and head and neck tumors and adjacent normal tissue. Nuclear extracts were mixed with γ^{32}P -end labelled double stranded oligonucleotides E_3AP-1 and 2ab, respectively and analyzing the formation of DNA-protein complexes by gel retardation assays. Twelve lung tumors were analysed and eight of them showed elevated levels of AP-1 activity as compared with either the adjacent normal tissue or the correspoding lymph nodes (Fig. 1). The same oligonucleotide hybrid, 2ab, was also used to test the presense of AP-1 activity in bladder and head and neck tumors. Nine out of fourteen bladder tumors tested showed elevated levels of AP-1 activity as compared to adjacent normal tissue (Fig. 2). Seven head and neck tumors seven tumors were analysed and all of them showed elevated AP-1 as compared with adjacent normal tissues (Fig. 3). A comparison of the data on the AP-1 activity and the pTNM in lung, bladder and head and neck tumors are shown in Tables I, II and III.

Having established that most of the examined lung and bladder tumors and all of the head and neck tumors had elevated AP-1 activity, we subsequently confirmed that the remaining non-tested AP-1-like sites (1ab,4ab) on HIV-1 LTR are functional. This was tested by employing nuclear extracts from lung tumor No 12 and bladder tumor No 1, mixed with a γ^{32}P-end labelled 2ab or 1ab oligonucleotide hybrids and employing the non-labelled oligonucleotide hybrids as competitors 1ab, 2ab, 3ab, 4ab, E_3AP-1 and analysing the formation of DNA-protein complexes by gel retardation assays. As shown in Figs. 4 (lung tumor) and 5 (bladder tumor) the non-labelled oligonucleotide hybrids E_3AP-1, 1ab, 2ab and 4ab competed with the labelled oligonucleotide hybrid 2ab for DNA-AP-1 protein complex formation. Also as shown in Fig. 6 (lung tumor) the non-labelled oligonucleotide hybrids 1ab, 2ab and E_3AP-1 competed with the labelled oligonucleotide hybrid 1ab for

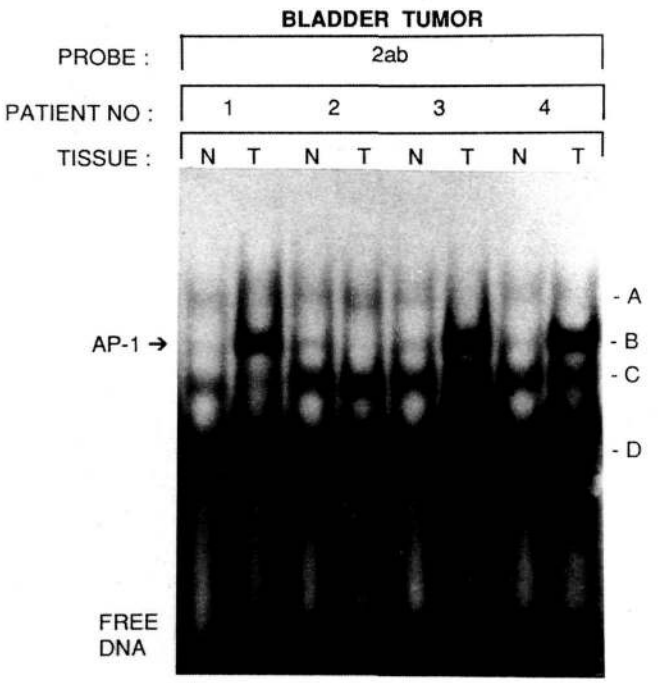

Figure 2. Electrophoretic mobility shift analysis using 2ab-AP-1 binding consensus oligonucleotide. 2000 cpm γ^{32}P-oligo was mixed with 20 µg of nuclear extracts from bladder tumors and adjacent normal tissues. The DNA-AP-1 protein complex is indicated by the arrow. N = normal, T = tumor.

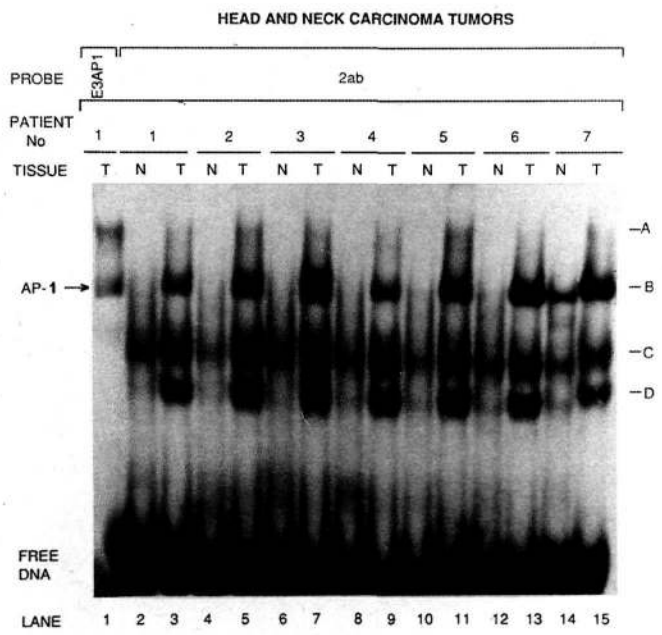

Figure 3. Electrophoretic mobility shift analysis using E_3AP-1 and 2ab-AP-1 binding consensus oligonucleotide. Nuclear extracts from head and neck carcinomas were incubated with γ^{32}P-end-labelled E_3AP-1 and 2ab-AP-1 oligonucleotide hybrids (lanes 1 and 2-15, respectively). The DNA-AP-1 protein complex is indicated by the arrow. N = normal, T = tumor.

DNA-AP-1 protein complex formation. This is not surprising since E_3AP-1 contains the AP-1 binding consensus from the E_3 promoter (37), while 1ab and 4ab are highly homologous (71.5%) to the same sequence (34). However, the non-labelled oligonucleotide hybrid 3ab did not compete for AP-1 binding activity with the labelled oligonucleotide hybrids 2ab and 1ab, presumably because it shares only a limited homology (57.1%) to the AP-1 concensus.

The non-labelled oligonucleotide hybrids E_3AP-1, 1ab, 2ab and 4ab competed with the labelled oligonucleotide

Table I. pTNM, AP-1 level and stage of differentiation in tumors of lung cancer patients.

Patient No[a]	pTNM[b]	AP-1 level[c]	Stage of differentiation
1	$T_3N_1M_0$	elevated	III
2	$T_2N_1M_0$	elevated	II
3	$T_2N_0M_0$	non-elevated	I
4	$T_2N_0M_0$	elevated	III
5	$T_1N_0M_0$	non-elevated	I
6	$T_3N_2M_0$	elevated	III
7	$T_2N_2M_0$	elevated	III
8	$T_4N_2M_0$	elevated	II
9	$T_2N_1M_0$	elevated	II
10	$T_2N_0M_0$	non-elevated	I
11	$T_2N_0M_0$	elevated	II
12	$T_2N_1M_0$	elevated	III

[a]All tissue specimens were obtained from lung cancer patients. [b]pTNM was defined according to the UICC classification. [c]Tumor/adjacent normal tissue.

Table II. pTNM, AP-1 level and stage of differentiation in tumors of bladder cancer patients.

Patient No[a]	pTNM[b]	AP-1 level[c]	Stage of differentiation
1	$T_3N_0M_0$	elevated	III
2	$T_0N_0M_0$	non-elevated	I
3	$T_2N_0M_0$	elevated	II
4	$T_3N_0M_0$	elevated	III
5	$T_3N_0M_0$	elevated	III
6	$T_1N_0M_0$	elevated	II
7	$T_0N_0M_0$	non-elevated	I
8	$T_0N_0M_0$	non-elevated	I
9	$T_1N_0M_0$	elevated	II
10	$T_0N_0M_0$	non-elevated	I
11	$T_2N_0M_0$	elevated	II
12	$T_0N_0M_0$	non-elevated	I
13	$T_1N_0M_0$	elevated	II
14	$T_2N_0M_0$	elevated	II

[a]All tissue specimens were obtained from bladder cancer patients. [b]pTNM was defined according to the UICC classification. [c]Tumor/adjacent normal tissue.

Table III. pTNM, AP-1 level and stage of differentiation of head and neck tumors.

Patient No[a]	pTNM[b]	AP-1 level[c]	Stage of differentiation
1	$T_3N_0M_0$	elevated	II
2	$T_3N_1M_0$	elevated	III
3	$T_2N_2M_0$	elevated	IV
4	$T_3N_1M_0$	elevated	III
5	$T_2N_0M_0$	elevated	II
6	$T_2N_0M_0$	elevated	II
7	$T_2N_0M_0$	elevated	II

[a]All tissue specimens were obtained from head and neck cancer patients. [b]pTNM was defined according to the UICC classification. [c]Tumor/adjacent normal tissue.

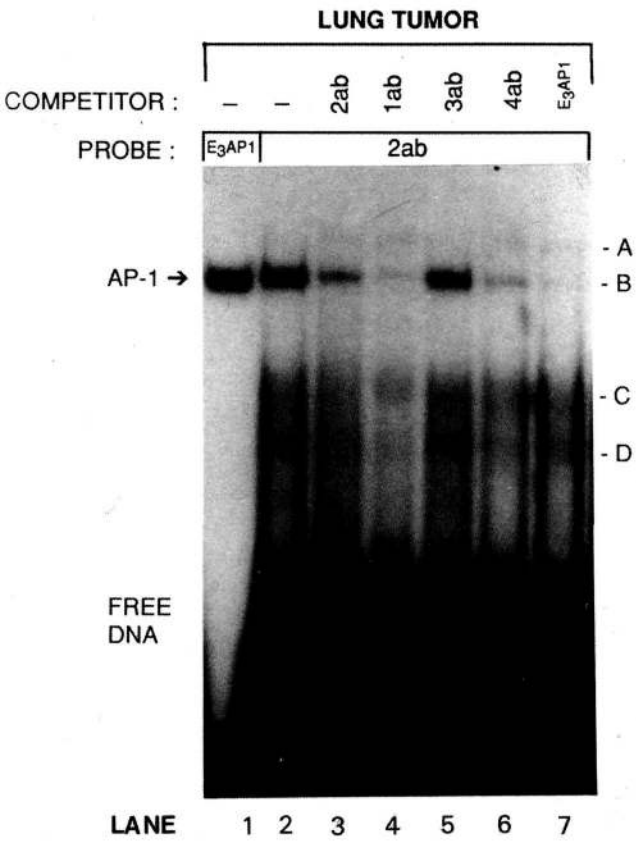

Figure 4. Effect of competitor sequences on gel electrophoretic mobility shift. Nuclear extracts from lung tumors were incubated with γ^{32}P-end labelled E_3AP-1 and 2ab-AP-1 oligonucleotide hybrids, respectively (lanes 1 and 2-7). In competition assays, 200-fold excess of cold competitor oligonucleotides 2ab, 1ab, 3ab, 4ab and E_3AP-1 (lanes 3-6 and 7, respectively), were incubated with the nuclear extracts before adding the probe. The DNA-AP-1 protein complex is indicated by the arrow.

E_3AP-1 for DNA-AP-1 protein complex formation (Fig. 7, lung tumor). In Fig. 8 the effect of JUN antibody on the formation of the DNA-protein complex between E_3AP-1 or 2ab oligo and the AP-1 protein from bladder tumor nuclear extracts is shown.

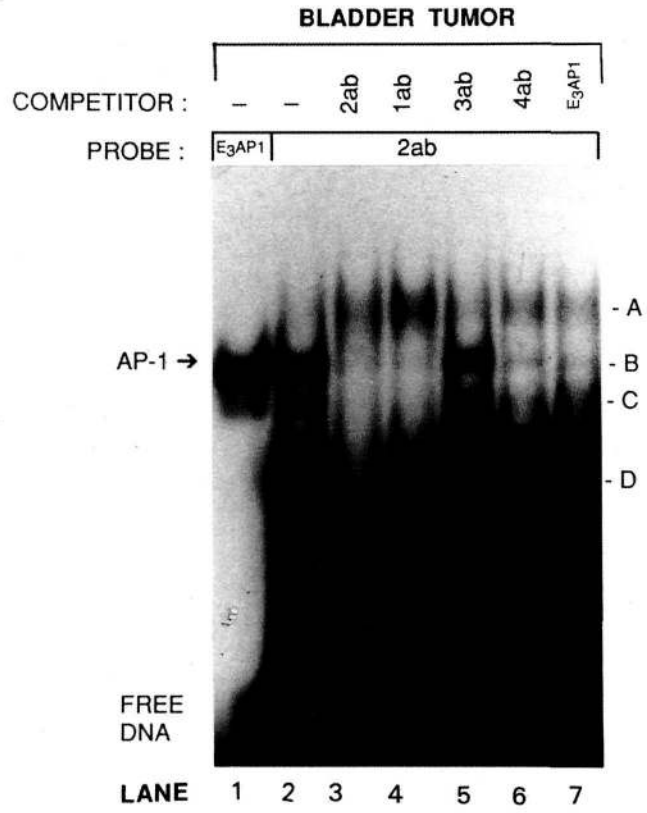

Figure 5. Effect of competitor sequences on gel electrophoretic mobility shift. Nuclear extracts from bladder tumors were incubated with γ^{32}P-end labelled E_3AP-1 and 2ab-AP-1 oligonucleotide hybrids, respectively (lanes 1 and 2-7). In competition assays, 200-fold excess of cold competitor oligonucleotides 2ab, 1ab, 3ab, 4ab and E_3AP-1 (lanes 3-7) were incubated with the nuclear extracts before adding the probe. The DNA-AP-1 protein complex is indicated by the arrow.

Discussion

The human immunodeficiency virus type 1 long terminal repeat, HIV-1 LTR, contains binding sites for several cellular transcription factors which contribute to HIV-1 gene expression. The interaction between cellular and viral proteins and DNA sequences of the HIV-1 LTR is proposed to play a crucial role in transactivating HIV-1 and in inducing the switch from latency to virus production and phenotypic appearance of cytopathic effects.

A number of studies have indicated that the AP-1 site is an important component of several regulatory regions. Functionally important AP-1 sites have been identified in the enhancer of the chicken ß-globin (38), metallothionein IIa (39) and collagenase genes (40); in the polyomavirus enhancer (41), and in the human ß-globin dominant control region (42). Furthermore, AP-1 binding sites have also been identified in the genome of the visna virus closely related to HIV-1 where they play a critical role in basal activity and transactivation of the viral LTR by the virally encoded TAT protein (43).

In a previous study we identified three newly elucidated AP-1 binding sites in the HIV-1 LTR using in gel retardation assays nuclear extracts from MDA MB 468 and HeLa tumor cells (34). In the present study we examined the gel mobility shift assays with the same synthetic oligonucleotides

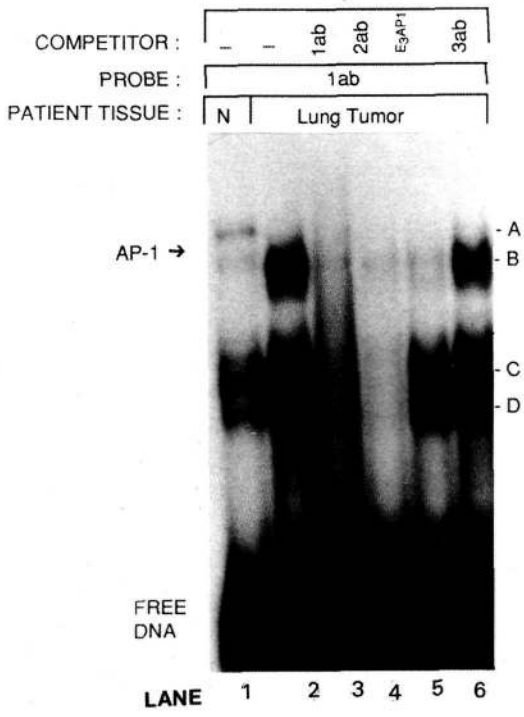

Figure 6. Effect of competitor sequences on gel electrophoretic mobility shift. Nuclear extracts from lung tumors and adjacent normal tissues (lanes 2-6 and 1, respectively), were incubated with γ^{32}P-end labelled 1ab-AP-1 oligonucleotide hybrid. In competition assays, 200-fold excess of cold competitor oligonucleotides 1ab, 2ab, E_3AP-1 and 3ab (lanes 3-6), were incubated with the nuclear extracts before adding the probe. Only the specific retarded bands of interest are shown.

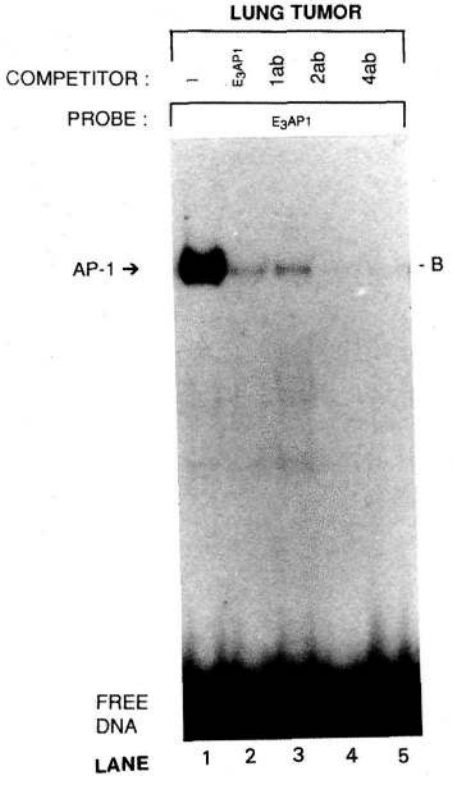

Figure 7. Effect of competitor sequences on gel electrophoretic mobility shift. Nuclear extracts from lung tumors were incubated with γ^{32}P-end labelled E_3AP-1 oligonucleotide hybrid. In competition assays, 200-fold excess of cold competitor oligonucleotides E_3AP-1 1ab, 2ab and 4ab (lanes 2-5) were incubated with the nuclear extracts before adding the probe. Only the specific retarded bands of interest are shown.

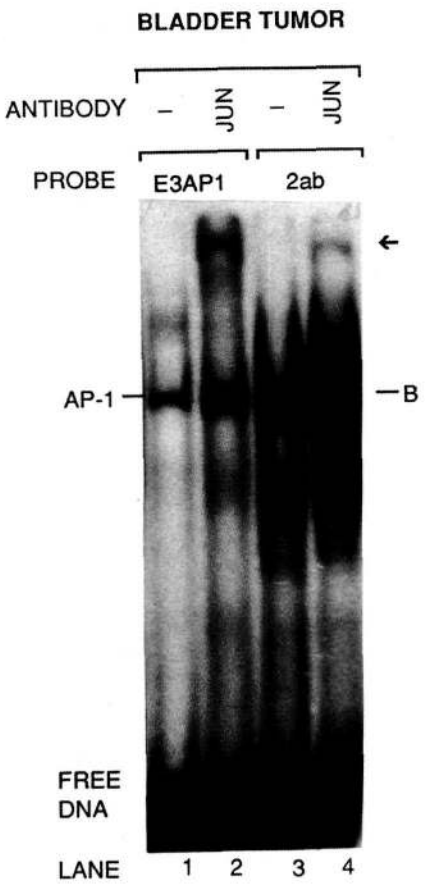

Figure 8. Effect of JUN antibody on the AP-1/oligonucleotide complexes studied by gel electrophoretic mobility shift assays. Nuclear extracts from bladder tumors were incubated with E_3AP-1 oligonucleotide (lane 1) and 2ab oligonucleotide (lane 3). JUN antibody and nuclear extracts from bladder tumors were incubated with E_3AP-1 oligonucleotide (lane 2) and 2ab oligonucleotide (lane 4), respectively. The shift in the mobility of the JUN complex is shown.

containing the putative HIV-1 LTR AP-1 sites and nuclear extracts from human lung, bladder and head and neck tumors. Several studies have suggested that AP-1 activity may be important in cell regulation and growth (44,45). In the present study we report that AP-1 activity is elevated in 75% of lung tumors, 66.6% of bladder tumors and in 100% of head and neck carcinomas examined as compared to adjacent normal tissue. These and other results (46-48) suggest that the products of *jun* and *fos* oncogenes represent a class of inducible cellular proteins that could modulate the expression of genes containing AP-1 binding sites since previous studies have demonstrated that there is elevated expression of the oncogenes H-*ras* and K-*ras* in the development of squamous cell carcinoma of the head and neck (49,50). It is therefore plausible that the increased levels of AP-1 we have observed in breast, bladder, lung and head and neck human tumors (47-51) are due to *ras* activation which triggers *fos* (52) and JUN/AP-1 (53) gene expression. Therefore, it would be of interest to examine whether a correlation between *ras, jun* and *fos* expression and tumor stage exists.

A comparision of AP-1 activity and pTNM was carried out for lung, bladder and head and neck tumors (Table I, II and III) as described previously in studies for human lung and bladder tumors (48). Although the number of samples

analysed was relatively small, a correlation was found between the stage of differentiation and AP-1 levels in head and neck tumors. The higher the stage of differentiation the higher the AP-1 levels (48).

The importance of AP-1 in the regulation of HIV-1 gene expression can only be speculated on. Many agents that induce transcription of latent, proviral DNA or reporter gene linked to the HIV-1 LTR (6-16) also induce a substantial level of *fos* expression (54,55). Although no causal link between these two phenomena has been demonstrated it is possible that AP-1 family proteins which represent a class of inducible cellular proteins could play a role in the transcriptional regulation of human immunodeficiency virus type 1 gene expression.

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