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AP-1 recognizes sequence elements on HIV-1 LTR in human epithelial tumor cell lines

V. ZOUMPOURLIS[1,2], M. ERGAZAKI[1,2] 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, Creece

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Abstract. Investigation of the nucleotide sequence of the HIV-1 LTR showed the presence of four novel short DNA regions which are homologous to the recognition site for the cellular transcription factor AP-1. Four short oligonucleotide hybrids containing these potential AP-1 sites were constructed and used in gel retardation assays and in competition experiments in order to determine the role of the AP-1 protein in the regulation of HIV-1 expression. The breast MDA MB 468 and cervical HeLa tumor cell lines, which are known to overexpress the AP-1 protein were used in a gel retardation assay as a control to study the affinity of the AP-1 to synthesized oligonucleotide sequences. We have observed specific binding of nuclear factor AP-1 to three of these oligonucleotide hybrids. These results demonstrate the presence of three novel AP-1 binding sites on HIV-1 LTR, one of which was found within the TAR element and in the Tat protein binding region. Moreover, they suggest that AP-1 could be contributing to HIV-1 transcriptional regulation through its interaction with the AP-1 binding sites of HIV-1 LTR.

Introduction

The human immunodeficiency virus type 1 (HIV-1) is the etiologic agent and the primary cause of AIDS (1-3). The expression of the virus is regulated both at transcriptional and post-transcriptional levels by several human and viral proteins (4-6). Control of HIV-1 transcription is mediated by *cis*-acting elements located in the viral long terminal repeats (LTRs), by the viral trans-regulatory protein Tat, and by cellular transcription factors which are constitutively expressed in most cells (e.g. NF-κB and NFAT-1) (7). *cis* elements include the negative regulatory element (NRE, located in the region between nucleotide positions -357 to -185 relative to the transcription initiation site +1), the enhancer (-103 to -81), the Sp1 element (-75 to -47), and the TATA box (-28 to -24). The *trans*-regulatory element TAR is located between residues -17 to +80. Various cellular proteins have been found to interact with the *cis* elements, such as AP-1 (8) and USF (9) with NRE; EBP-1 with the enhancer (10); Sp1 with the sequence motif Sp1 (11) and TFIID with the TATA box (12). The viral protein Tat interacts with the *trans*-regulatory element TAR (5,13,14). The AP-1 binding site was initially described in the enhancer elements of the Simian Virus 40 promoter and the human metallothionein IIA promoter (15). A group of polypeptides originally purified from HeLa cells was designated as transcription factor AP-1 on the basis of DNA binding specificity and *in vitro* transcriptional analysis (16). The proteins named AP-1 in fact represent a family of transcription factors encoded by the members of the *jun* and *fos* multigene families able to bind as homo- and/or heterodimers to the AP-1 consensus (17,18).

Two binding sites for transcription factor AP-1 have been mapped within the NRE of HIV-1 LTR. Sequences between nucleotides -348 to -343 and -336 to -331 are similar to HeLa cell AP-1 binding sites and have been shown to interact with the FOS-complex and FOS-related antigens (8). In this study we have further examined the HIV-1 LTR, and we have identified three additional AP-1 binding sites one of which was found within the TAR element. According to this result, AP-1 could be contributing to the HIV-1 transcriptional regulation through its interaction with AP-1 binding sites of HIV-1 LTR.

Materials and methods

Cells and culture conditions. Human MDA MB 468 breast and HeLa cervical epithelial tumor cells were grown exponentially in Ham's medium containing 10% fetal calf serum and used for the preparation of nuclear extracts.

Preparation of cell extracts. The tumor cell lines were homogenized in 2 ml hypotonic buffer (25 mM Tris-HCl pH 7.5 KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF) at 5-10 mg/ml. The nuclei were pelleted at 2500 rpm in a Sorvall SS34 rotor for 10 min at 4˚C. The pellets were washed 3

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

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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. Supernatant was removed and the extracts were stored at -70°C. Protein estimation was performed as described by Bradford (19).

Preparation of double stranded oligonucleotide hybrids. Eight single stranded DNA oligonucleotides were made on an Applied Biosystems 381A DNA synthesizer. These were:

1a AGCTTAAGACCAATGACTTACAAGGCAGCA
1b ATTCTGGTTACTGAATGTTCCGTCGTTCGA
2a AGCTTCTAGTACCAGTTGAGCCAGAGAAGTTA
2b AGATCATGGTCAACTCGGTCTCTTCAATTCGA
3a AGCTTCATGGAATGGATGACCCGGAGAGAGAA
3b AGTACCTTACCTACTGGGCCTCTCTCTTTCGA
4a AGCTTAGACCAGATCTGAGCCTGGGAGCTCTCTTA
4b ATCTGGTCTAGACTCGGACCCTCGAGAGAATTCGA

The oligonucleotides were removed from the synthesis column by elution with 3x1 ml of ammonia. This solution was incubated at 55°C overnight to deprotect the oligonucleotides. To further purify the oligonucleotides, an Applied Biosystems oligonucleotide purification cartridge (OPC) was used. To anneal complementary single stranded oligonucleotides (i.e. 1a to 1b) they were both incubated at a concentration of 0.05 M in TE. The solution was then heated to 90°C and allowed to cool slowly to less than 30°C. This results in the formation of double stranded oligonucleotide hybrids. To check the succession of the annealing the double stranded oligonucleotide hybrids were run on an 8% polyacrylamide gel alongside the single-stranded oligonucleotides.

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* (20).

Oligonucleotide labelling. The above described oligonucleotide hybrids and the oligonucleotide E_3AP-1 representing the region between nucleotides -81 and -103 at the E1A-inducible E_3 promoter (21) were labelled with γ^{32}P-ATP using T4 polynucleotide kinase from Boehringer. The oligos were incubated sequentially at 37°C for 30 min, at 70°C for 5 min, at 37° for 10 min, at RT for 5 min and on ice for 5 min.

Gel retardation assays. DNA binding reactions were carried out as follows: 2000 cpm γ^{32}P-oligo were mixed with nuclear proteins (20 µg) in binding buffer (50 mM Hepes pH 8.0, 500 mM NaCl, 0.5 mM PMSF, 0.5 mg/ml BSA, 20% glycerol, 1 mM EDTA) plus 1 mM DTT and 150 µg/ml poly(dI-dC). The reaction mixture was left for 30 min at 0°C. Samples were subjected to electrophoresis on 5% polyacrylamide gels, dried and exposed to X-ray film (RX Fuji, Japan).

A rabbit polyclonal antibody to the human JUN protein (a gift from Dr D. Gillespie, The Beatson Institute, Glasgow, UK) was employed in gel mobility shift assays.

Results

The HIV-1 LTR contains four short DNA regions (1ab to 4ab) which are homologous to the recognition site for the cellular transcription factor AP-1 (Fig. 1). From these four sequences, the AP-1 like sequence in the region 2ab shows the highest homology (85.8%) to the AP-1 consensus and therefore the corresponding oligonucleotide hybrid 2ab was initially used to test the affinity of the AP-1 protein in MDA MB 468 and HeLa tumor cell lines. This was achieved by preparing nuclear extracts, mixing them with γ^{32}P-end labelled double stranded oligonucleotides E_3AP-1 and 2ab and analysing the formation of DNA-protein complexes by gel retardation assays. In addition, we also examined whether the remaining non-tested AP-1-like sites on HIV-1 LTR are functional. In Fig. 2 is shown a panel of lanes with competition experiments between 2ab end-labelled oligonucleotide and the representative set of AP-1-like sites in HIV-1 LTR (1ab or 2ab or 3ab or 4ab) oligonucleotides. These competition reactions were performed on the MDA MB 468 tumor cell line. The 1ab, 2ab and 4ab oligonucleotides compete to a different extent depending on their homology to AP-1 consensus (see Fig. 1) for the labelled 2ab oligonucleotide. This is not surprising since 1ab and 4ab are highly homologous (71.5% to the E_3AP-1 which contains the AP-1 binding consensus from the E_3 promoter) (21), (Fig. 1). However, the non-labelled oligonucleotide hybrid 3ab did not compete for AP-1 binding activity with the labelled oligonucleotide hybrid 2ab, presumably because it shares only a limited homology (57.1%) to the AP-1 consensus.

To test further whether these putative AP-1 binding sites are functional, the control end-labelled oligonucleotide hybrid E_3AP-1 was used in competition reactions with 1ab, 2ab 3ab, 4ab or non-labelled E_3AP1 oligonucleotide in nuclear extracts from the MDA MB 468 tumor cell line. As shown in Fig. 3 non-labelled oligonucleotides E_3AP-1, 2ab and 4ab compete well, 1ab compete weakly and 3ab does not compete to the labelled oligonucleotide E_3AP-1 for DNA-AP-1 protein complex formation. The oligonucleotide hybrid 2ab was then used to test the presence of AP-1 activity in HeLa cells. Competition experiments (Fig. 4, lanes 3 to 5) showed that in all cases non-labelled 1ab, 2ab and 4ab oligonucleotides compete for end-labelled 2ab oligonucleotide.

We have shown the effect of JUN antibody on the formation of the DNA-protein complex between E_3AP-1 and the AP-1 protein from HeLa nuclear extracts. In Fig. 5 is shown the effect of JUN antibody on the formation of the DNA-protein complex between E_3AP-1 and the AP-1 protein in HeLa nuclear extracts (lane 6). Also in the same Figure is shown that the non-labelled oligonucleotide hybrids 1ab or, 2ab and 4ab competed with the labelled oligonucleotide hybrid E_3AP-1 for DNA-AP-1 protein complex formation (lanes 3-5), therefore suggesting that these binding sites are likely to be functional.

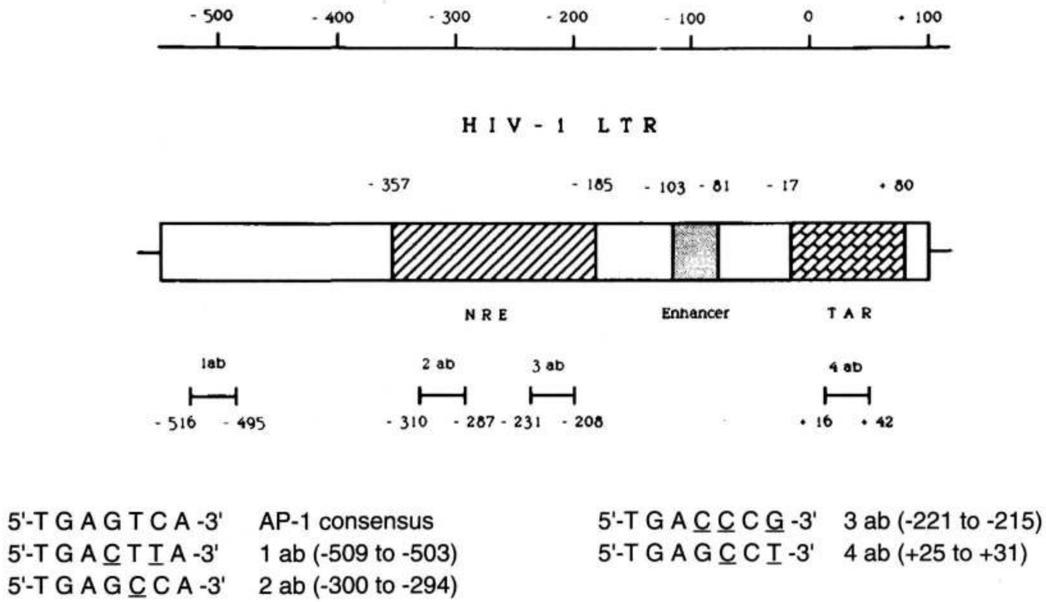

Figure 1. Schematic representation of the HIV-1 LTR. The locations of NRE, enhancer and TAR sequences are shown. The nucleotide sequence of AP-1 consensus is shown as well as the nucleotide sequences of putative AP-1 sites located in the HIV-1 LTR alongside their nucleotide position. Nucleotide differences between AP-1 consensus and HIV-1 LTR AP-1 sequences are underlined.

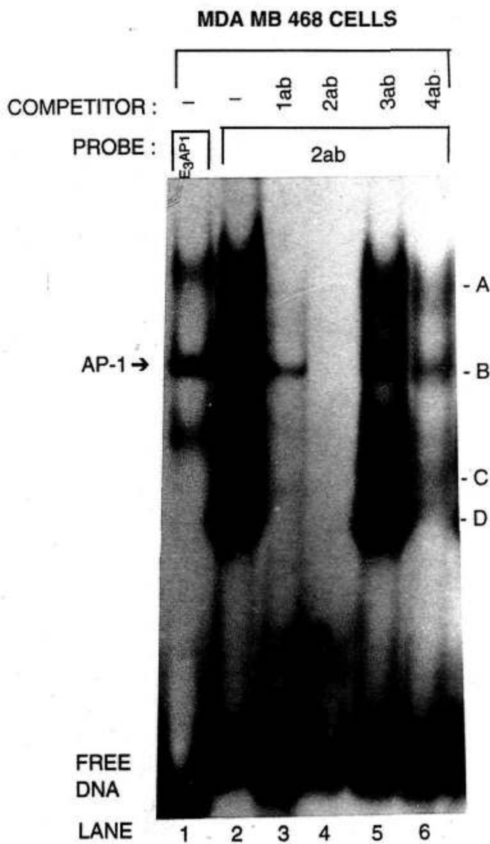

Figure 2. Effect of competitor sequences on gel electrophoretic mobility shift. Nuclear extracts from MDA MB 468 cells were incubated with $\gamma^{32}P$-end labelled E_3AP-1 and 2ab-AP-1 oligonucleotide hybrid (lanes 1 and 2-6, respectively). In competition assays, 200-fold excess of cold competitor oligonucleotides 1ab, 2ab, 4ab and 3ab (lanes 3-6), were incubated with the same nuclear extracts. The DNA-AP-1 protein complex is indicated by the arrow.

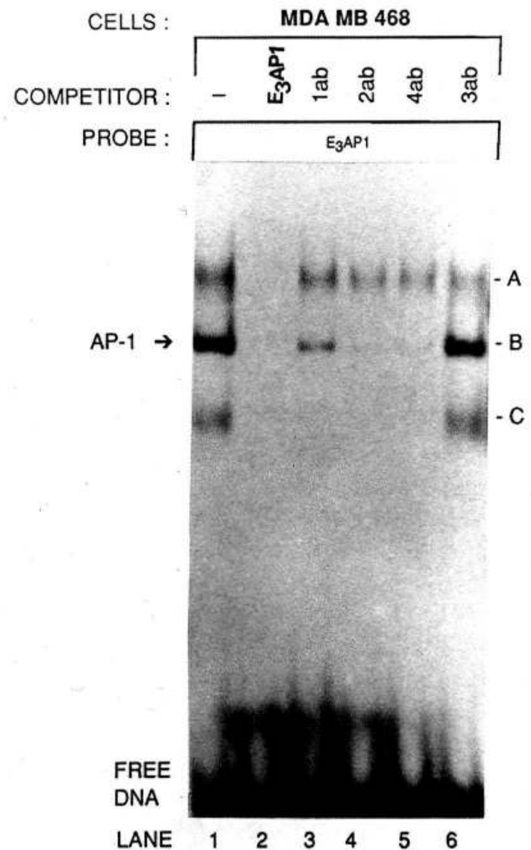

Figure 3. Effect of competitor sequences on gel electrophoretic mobility shift. Nuclear extracts from MDA MB 468 tumor cells were incubated with $\gamma^{32}P$-end labelled E_3AP-1 oligonucleotide hybrid. In competition assays, 200-fold excess of cold competitor oligonucleotides E_3AP-1 and 1ab, 2ab, 4ab and 3ab (lanes 2 and 3-6, respectively), were incubated with the nuclear extracts before adding the probe. The DNA-AP-1 protein complex is

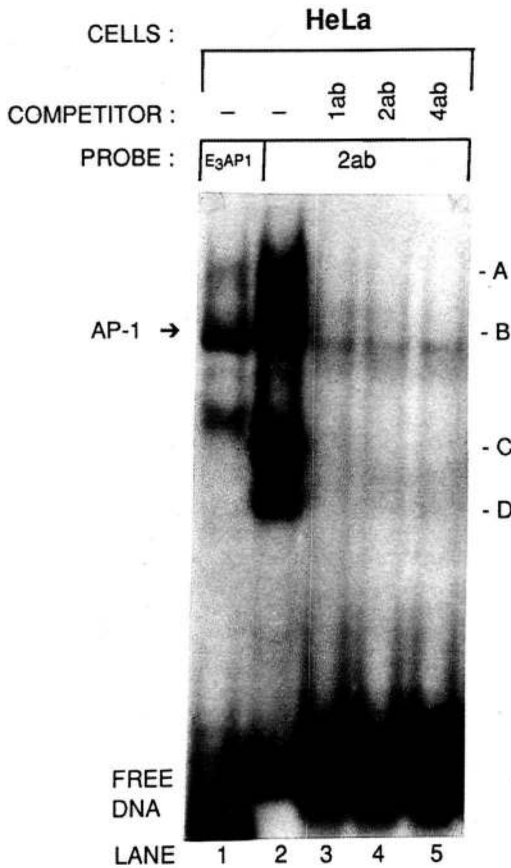

Figure 4. Effect of competitor sequences on gel electrophoretic mobility shift. Nuclear extracts from HeLa cells, were incubated with γ^{32}P-end labelled E_3AP-1 and 2ab-AP-1 oligonucleotide hybrids (lanes 1 and 2-5, respectively). In competition assays, 200-fold excess of cold competitor oligonucleotides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

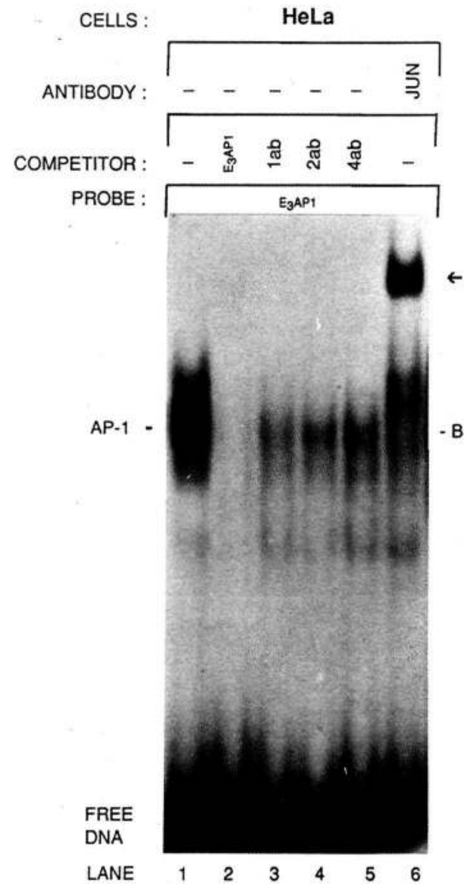

Figure 5. Effect of competitor sequences and JUN antibody on the AP-1/ oligonucleotide complexes analyzed by gel electrophoretic mobility shift. Nuclear extracts from HeLa cells incubated with γ^{32}P-end labelled E_3AP-1 oligonucleotide hybrid. In competition assays, 200-fold excess of cold competitor oligonucleotides 1ab, 2ab, 4ab and E_3AP-1 (lanes 2-4 and 5, respectively), were incubated with the nuclear extracts before adding the probe. JUN antibody and nuclear extracts from HeLa cells were incubated with E_3AP-1 oligonucleotide (lane 6). The shift in the mobility of the JUN complex is indicated by the arrow.

Discussion

In this study we found that the HIV-1 LTR contains three novel AP-1 binding sites. One of the three novel AP-1 sites was found, within the TAR region, one within NRE and the other further upstream. All three sites are functional since their corresponding oligonucleotide hybrids compete one another as well as the oligonucleotide hybrid E_3AP-1 which contains the AP-1 binding site of the E_3 inducible promoter. The potential transcriptional regulation of HIV-1 gene expression by the AP-1 binding site within NRE is further supported by the fact that Curran and his colleagues have identified two additional AP-1 sites in this LTR region (8). However, the novel AP-1 site we found within the TAR region appears to be of even greater interest. Maximal expression of proviral HIV-1 DNA is attributed mainly to the Tat protein, which is thought to function at the transcriptional level through a nascent RNA copy of the TAR region. According to Berkhout and his colleagues the Tat protein could target the LTR transcriptional unit directly bypassing the use of TAR RNA (22). In this instance it is possible that AP-1 protein could participate in this mechanism through TAR DNA sequence. Further experiments are needed to test this hypothesis.

Although our study demonstrated novel AP-1 binding sites in the HIV-1 LTR sequences, further studies would be required to establish a functional relationship of these HIV LTR AP-1 binding sites and the elevated AP-1 levels in the HIV life cycle.

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