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Analysis of SRF, SAP-1 and ELK-1 transcripts and proteins in human cell lines

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Abstract We have analysed the expression of the genes encoding transcription factors involved in *c-fos* transcriptional regulation, i.e. the serum response factor (SRF) and the ETS-related proteins ELK-1 and SAP-1, in a variety of human cell lines. RNA was determined by Northern blot analysis, and proteins were detected on Western blots: the two analyses gave essentially identical results. SRF was expressed at similar levels in all cell lines tested. In contrast, SAP-1 and ELK-1 expression varied from one cell line to another. Interestingly, in any given cell line, high levels of one protein were accompanied by low levels of the other. Similar results were obtained by electromobility shift assays (EMSA), using antibodies directed against the proteins. Thus, our data raise the possibility of a coordinated regulation of the expression of these two Ets genes, at both RNA and protein levels.

Key words: Serum responsive element; Serum responsive factor; SAP-1; ELK-1

1. Introduction

The *c-fos* proto-oncogene is an immediate early gene which is transiently transcribed in response to growth factors and other stimuli [1]. *c-fos* transcriptional activation by growth factors seems to be universal. In all cell types tested, from fibroblasts [2] to lymphocytes [3] and nerve cells [4], the serum responsive element (SRE), a DNA element in the *c-fos* promoter, is responsible for most *c-fos* responses. Several proteins bind to the SRE [5], including $p67^{SRF}$, which recognizes a CArG box in the center of the element [6,7], and $p62^{TCF}$, which binds to an Ets box located in the SRE in close proximity to the CArG box [8,9]. While binding of $p62^{TCF}$ is weak, $p62^{TCF}$ recognized the SRE with a high affinity in the presence of SRF, allowing the formation of a ternary complex. $P62^{TCF}$ has been shown to be highly related to the ETS protein ELK-1 [10]. Proteins from another sub-group in the ETS family, the SAP-1 proteins, are also able to form a ternary complex and display a TCF activity [11]. It has been shown that there are two separate signalling pathways for the activation of distinct TCFs: one dependent on Ras and MAP kinase and converging on TCF/ELK-1, and the other targeting TCF/SAP-1 independently of Ras and MAP Kinase, suggesting that these two TCFs do not share exactly the same function in the cell. Some external activating signals are mediated through TCF phosphorylation [10,12], whereas other signals go through the SRF protein itself [13,14].

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Abbreviations: SRE, serum responsive element; SRF, serum responsive factor; TCF, ternary complex factor

The pattern of SRF gene expression has been described in the human cell line HeLa [15] and in murine myoblastic cell lines [16]. SAP-1 and ELK-1 transcripts have been analyzed in human and mouse tissues with discordant results: Giovane et al. [17] have reported distinct tissue specificities for murine ELK-1 and SAP-1a, SAP-1a being expressed at a high level in lung and liver, whereas ELK-1 was strongly expressed in heart and brain but undetectable in lung. In contrast, Rao et al. [18] showed that, in mouse embryo, ELK-1 was only expressed in lung and liver, its expression in adult tissues being restricted to testis, lung and, to a lesser extent, brain. Finally, SAP-1a and ELK-1 have more recently been described as being expressed at similar levels in a great variety of human tissues [19]. At the protein level, Pingoud et al. [20] did not detect any SAP-1 in ternary complex formed by nuclear proteins of HeLa cells, and ELK-1 appeared to be responsible for most of the TCF activity in these cells.

Here, we have used Northern and Western analyses, followed by mRNA and protein quantification, to study the expression of SRF, SAP-1a and ELK-1 in a variety of human cell lines from distinct tissue origins. Our results indicate (1) that SRF is expressed at similar levels in all cell lines tested, whereas SAP-1a and ELK-1 mRNA and protein levels are less homogeneous, and (2) that high levels of one TCF transcript and protein are accompanied by low levels of the other. These results were confirmed using EMSA analysis.

2. Materials and methods

2.1. Cell lines

Human cell lines used in this study included HeLa (a cervical carcinoma), Saos-2 (an osteosarcoma), IARC-EW17 (a Ewing sarcoma), K526 (an erythroleukemia), E418 (a lymphoblastoid EBV-positive B cell line), Ramos and BL2 cells (an EBV-positive and an EBV-negative Burkitt's lymphoma), and Jurkat (an acute T cell leukemia). They were maintained in RPMI 1640 or DMEM medium containing 10 or 15 % FCS.

2.2. Northern analysis

Total RNA was isolated by the acid guanidinium thiocyanate/cesium chloride method [21]. RNA (30 μg/lane) was subjected to electrophoresis in agarose containing 1.2% formaldehyde and transferred onto nitrocellulose [22]. Hybridization was performed at 42°C in 5×SSPE, 0.1% SDS, 5×Denhardt and 100 μg salmon sperm DNA, in the presence of 50% formamide. The following probes were prepared and labelled, using the Prime-a-gene system kit (Promega) and [α-^{32}P]dCTP:

SRF, a 450 bp *Hin*dIII-*Xho*I fragment from human SRF cDNA corresponding to the amino-terminal portion [15].

FLI-1, a 1410 bp fragment encompassing the entire ORF of the human FLI-1 cDNA: RT-PCR was performed from IARC-EW11 (a Ewing Sarcoma cell line) total RNA using the following primers: 5'ATTCGCTCTAGAATGTGTGGAATATTGGGG3' 5'ATTGCCTCTAGAAGCTTCTAGTAGTAGCTG3'. The PCR product corresponding to FLI-1 cDNA was cloned in pUC 18.

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SAP-1a, a 185 bp *Hin*dIII 3′ fragment from human SAP-1 cDNA [11].

ELK-1, a 516 bp *Apa*I 3′ fragment from human ELK-1 cDNA [23]. After overnight hybridization, blots were washed in 2×SSPE, 0.1% SDS (45°C, 15 min) followed by washing in 1×SSPE, 0.1% SDS (65°C, 20 min) and exposed to X-ray films for various periods of time.

Northern blot signals were quantified using a Bas 1000 Phosphoimager-(Mac Bas V1.01, Fuji) and 18S RNA levels as a control.

2.3. Nuclear extracts

Nuclear extracts were prepared according to the method described by Andrews and Faller [24].

2.4. Western blot analysis

Nuclear proteins (10 μg/lane) were loaded onto 7.5% SDS-PAGE gels (acrylamide 100:1) prior to electrophoretic transfer onto a PVDF nitrocellulose membrane (Millipore). The blots were blocked with 10% dry milk and incubated with a rabbit antiserum directed against SRF, ELK-1, or ELK-1 and SAP-1. Anti-SRF and anti-SAP-1/ELK-1 antibodies were raised against GST-fusion proteins prepared in bacteria. The blots were subsequently incubated for 1 h at room temperature with antirabbit horseradish peroxidase-conjugated antibodies, prior to exposure to the ECL substrate. Western blot signals were measured by scanning of the autoradiogram followed by quantification using Fuji Mac bas software.

2.5. Electromobility shift assays

EMSA were performed using 6 μg of protein and a radiolabeled wtETS SRE probe as previously described [25]. Samples were incubated for 15 min at room temperature with either an anti-SAP-1a goat polyclonal antibody directed against the C-terminal region of SAP-1a (aa 409–428) (Santa Cruz), or an anti-ELK-1 goat polyclonal antibody directed against the C-terminal region of ELK-1 (aa 407–428) (Santa Cruz).

3. Results and discussion

A panel of human cell lines was analysed by Northern analysis in order to monitor SAP-1, ELK and SRF expression.

A major ubiquitous transcript of approx. 4.5 kb was detected with the SRF probe in all cell lines tested (Fig. 1); identically high levels were observed in all samples. These results stand in contrast with data reported by Norman et

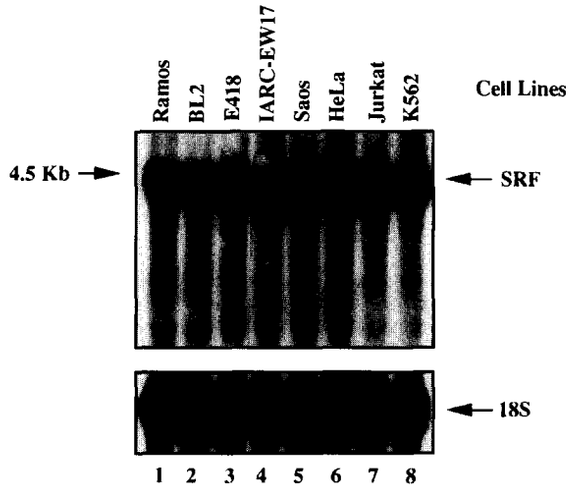

Fig. 1. SFR mRNA levels in various cell lines. 30 μg of total RNA from Ramos (lane 1), BL2 (lane 2), E418 (lane 3), IARC-EW17 (lane 4), Saos (lane 5), HeLa (lane 6), Jurkat (lane 7) and K562 (lane 8) cell lines were subjected to Northern blot analysis. Upper part: hybradization with the SRF probe; lower part: hybridization of the same blot with the control probe (18S RNA).

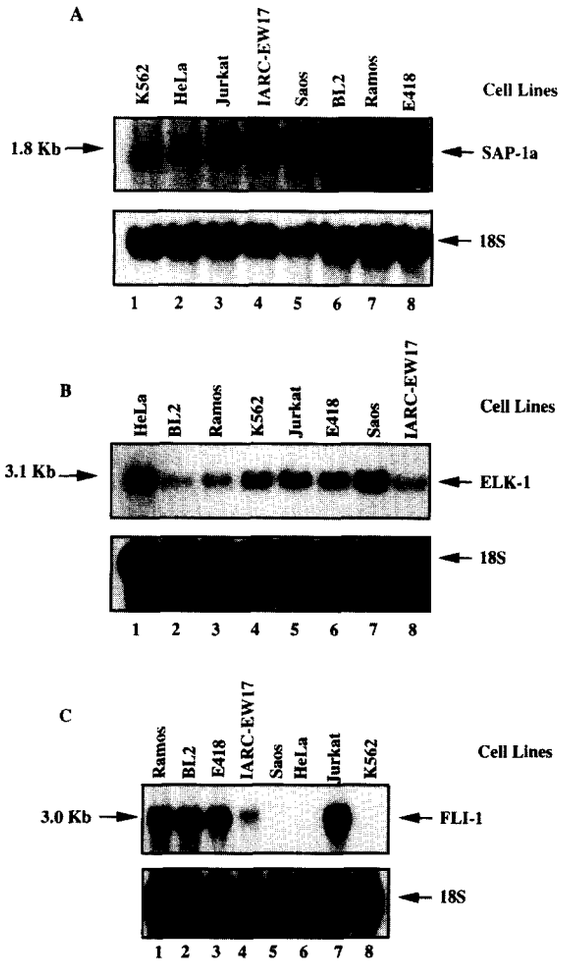

Fig. 2. TCF mRNA levels in various cell lines. (A) 30 μg total RNA from K562 (lane 1), HeLa (lane 2), Jurkat (lane 3), IARC-EW17 (lane 4), Saos (lane 5), BL2 (lane 6), Ramos (lane 7) and E418 (lane 8) cell lines were subjected to Northern blot analysis. Upper part: SAP-1a mRNAs; lower part: control probe (18S RNA). (B) 30 μg of total RNA from HeLa (lane 1), BL2 (lane 2), Ramos (lane 3), K562 (lane 4), Jurkat (lane 5), E418 (lane 6), Saos (lane 7) and IARC-EW17 (lane 8) cell lines were subjected to Northern blot analysis. Upper part: ELK1 mRNAs; lower part: control probe (18S RNA). (C) 30 μg of total RNA from Ramos (lane 1), BL2 (lane 2), E418 (lane 3), IARC-EW17 (lane 4), Saos (lane 5), HeLa (lane 6), Jurkat (lane 7) and K562 (lane 8) cell lines were subjected to Northern blot analysis. Upper part: FLI-1 mRNAs; lower part: control probe (18S RNA).

al. [15] who described two SRF mRNAs of 4.5 and 2.9 kb in HeLa cells. In their experiments, however, cells had been synchronized by serum deprivation and subsequently activated by growth factors, whereas we used exponentially growing cells. Thus, the 4.5 SRF transcript that we observed could be the major component of growing cells whereas the expression of the 2.9 kb species could be restricted to a short period at the onset of the G_1 phase. Indeed, the size of SRF transcripts could depend on the cell cycle, as suggested by data on terminal differentiation of myoblastic cell lines [16].

We next analysed TCF transcripts in the same cell lines: transcripts of approx. 1.8 and 3.1 kb were detected using a SAP-1a probe (Fig. 2A) and an ELK-1 probe (Fig. 2B), respectively. These transcripts were detected in all cell lines tested, suggesting that they are ubiquitously expressed. In

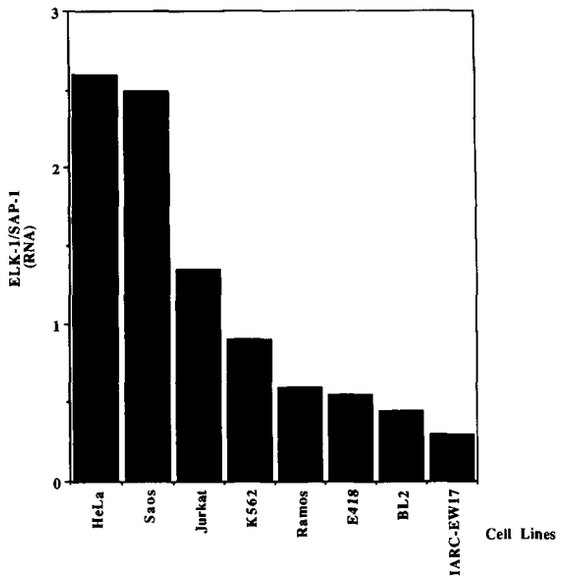

Fig. 3. Ratio between ELK-1 and SAP-1a mRNA levels in various cell lines. Hybridization to the SAP-1a and ELK-1 mRNAs was quantified on a Bas 1000 phosphoimager and standardized by reference to the 18S control probe. The ratio between ELK-1 and SAP-1a RNA expression level for each cell line is shown.

contrast, FLI-1, a member of the ETS family of DNA binding proteins known to be tissue-specific, was detected only in the four lymphocytic cell lines (Ramos, BL2, E418 and Jurkat), and not in K562, Hela, or Saos cells (Fig. 2C), Note that a faint band corresponding to the EWS-FLI1 fusion transcript of approx. 3.1 kb (vs. the normal 3.0 kb transcript FLI-1) is observed in the IARC-EW17 cell line, as expected for a cell line derived from a Ewing's sarcoma [26,27].

Taken together, our data suggest that, in contrast to FLI-1, SAP-1a and ELK-1 are ubiquitously expressed. However, SAP-1a and ELK-1 mRNA levels were less homogeneous than SRF mRNA levels. Heterogeneity of cell lines with regard to TCF mRNA expression was confirmed by quantification of the Northern blot signals on a Bas 1000 Phosphoimager using an 18S probe as an internal reference. Fig. 3 shows the ratio between ELK-1 and SAP-1 RNA levels for each cell line. For example, HeLa cells were among the cell lines which expressed the highest level of ELK-1 mRNA, but low levels of SAP-1 (in agreement with data at the protein level from Pingoud et al. [20]).

In thus appears that expression of SAP-1a and ELK-1 may be differentially controlled, whereas SRF is expressed at similar levels in all cell lines tested.

In order to verify these results, we have used Western blot analysis to study the expression of these genes at the protein level. SRF was detected at similar levels in all the cell lines, except for Jurkat, which expressed slightly higher levels of SRF (data not shown). We next used two antisera to detect SAP-1 and ELK-1 proteins, one of which crossreacts with both proteins (Fig. 4), the other being specific for ELK-1 (data not shown). SAP-1 and ELK-1 proteins were also present in all cell lines tested but were expressed in a heterogenous manner, as detected with both antibodies. Again, the Jurkat and here also the Ramos cell lines expressed higher levels of both SAP-1 and ELK-1 when compared to the other cell lines. In order to standardize the quantitative analysis among all cell lines, we used the anti-SAP-1 antibody which also recognizes ELK-1 (Fig. 4) for quantification, and the results were plotted as ratios between the signal intensity corresponding to ELK-1 protein and that corresponding to SAP-1a protein (Fig. 5).

Ratios between ELK-1 and SAP-1a protein levels were in agreement with the corresponding ratios at the RNA level,

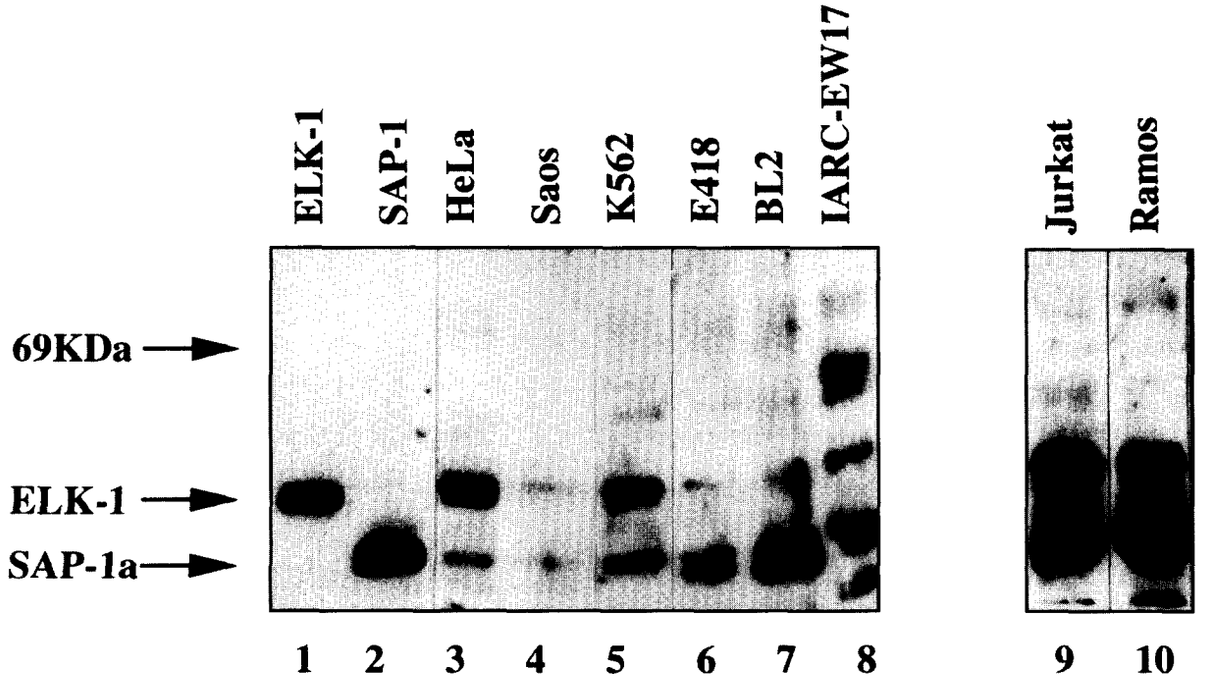

Fig. 4. TCF levels in various cell lines. About 10 μg of nuclear protein extracts from HeLa (lane 3), Saos (lane 4), K562 (lane 5), E418 (lane 6), BL2 (lane 7), IARC-EW17 (lane 8), Jurkat (lane 9) and Ramos (lane 10) cell lines and equivalent amounts of in vitro translated ELK-1 (lane 1) or SAP-1a (lane 2) as controls, were subjected to Western blot analysis, using an anti SAP-1/ELK-1 antiserum.

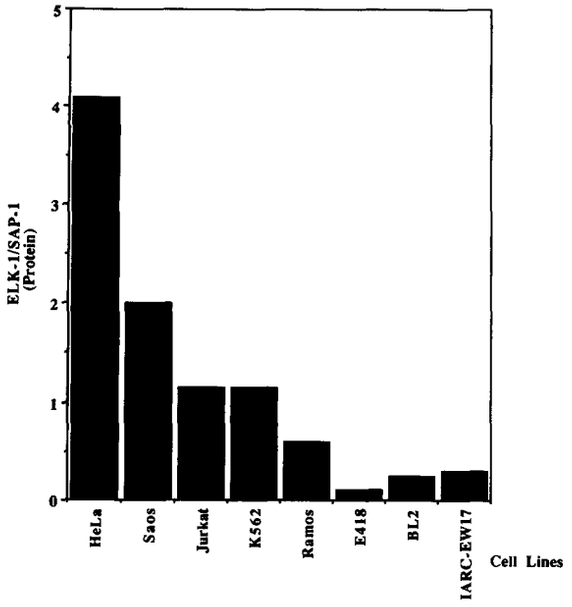

Fig. 5. Ratio between ELK-1 and SAP-1a protein levels in various cell lines. Signals for both SAP-1a and ELK-1 proteins, as revealed by Western blot analysis, were quantified (see Section 2). The ratio between ELK-1 and SAP-1a protein expression level for each cell line is shown.

including for the two cell lines (Jurkat and Ramos) showing a high absolute level of TCF proteins. HeLa cells, which showed the highest ratio between ELK-1 and SAP-1a mRNA levels, also displayed the greatest ratio between ELK-1 and SAP-1a at the protein level (Fig. 5).

In order to confirm these observations, we next analysed the composition of ternary complexes formed in nuclear extracts of some of these cell lines. EMSA was performed using specific and non-crossreacting anti-SAP-1a or anti-ELK-1 antibodies, which supershift the corresponding ternary complexes (Fig. 6A). Fig. 6B shows the results for two cell lines chosen for their high relative levels of expression of ELK-1(HeLa) or SAP-1a (BL2). The results were consistent with data obtained by Western and Northern analyses. Ternary complexes in the HeLa nuclear extract were supershifted by anti-ELK-1 antibodies, whereas the anti-SAP-1a antibody did not have any effect. In contrast, in BL2 nuclear extracts, the ternary complex was supershifted only by the anti-SAP-1a antibodies.

Taken together these results confirm the hypothesis that different cells express different relative levels of the two TCFs, ELK-1 and SAP-1, thus raising the possibility that they may be regulated in a distinctive manner and may exert distinct function in cells.

Indeed, two separate signalling pathways leading to modification of different TCFs have been described: TCF/ELK-1 would be a target for the Ras/MAP kinase pathway, whereas phosphorylated TCF/SAP-1 complexes can be detected in the absence of MAP kinase activation [28]. Furthermore, SAP-1 and ELK-1 have different patterns of sequence recognition [29], and we have recently shown that SAP-1a can transactivate the *c-fos* SRE in the absence of SRF, whereas ELK-1 cannot[1]. Therefore, it would not be surprising if SAP-1 and ELK-1, which seem to play distinct roles in the induction of immediate early genes, were found to be regulated differently.

Interestingly, our data also suggest the existence of an inverse correlation between the amounts of the two TCF related transcripts and proteins, SAP-1a and ELK-1: a high level of

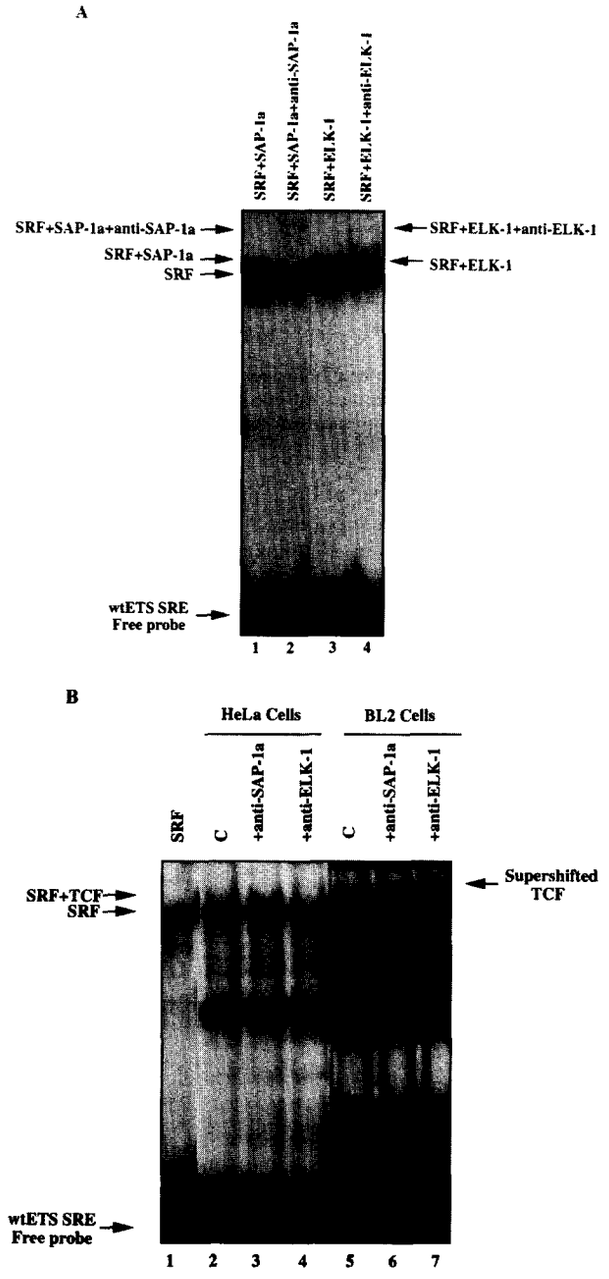

Fig. 6. Analysis of the composition of ternary complexes in HeLa and BL2 nuclear extracts. (A) Control: equivalent amounts of in vitro translated SAP-1a or ELK-1 proteins were analysed by EMSA in the presence of SRF, using either an anti-SAP-1a or an anti-ELK-1 antibody as indicated; SRF+SAP-1a (lane 1), SRF+SAP-1a+anti-SAP-1a (lane 2), SRF+ELK-1 (lane 3), SRF+ELK-1+anti-ELK-1 (lane 4). (B) In vitro translated SRF protein (lane 1), or 6 μg of nuclear extracts from HeLa cells (lanes 2–4) or BL2 cells (lanes 5–7) were analysed by EMSA using either an anti-SAP-1a (lanes 3,6) or anti-ELK-1 (lanes 4,7) antibody.

[1]Transactivation of *c-fos* SRE through SAP-1a in the absence of SRF. Masutani, H., Magnaghi-Jaulin, L., Groisman, R., Ait-SiAli, S., Robin, P. and Harel-Bellan, A. (submitted).

one TFC was accompanied by low levels of the other, at both the RNA and protein levels, raising the possibility that SAP-1 and ELK-1 could be expressed in a coordinated manner.

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