Transcriptional activity and constitutive nuclear localization of the ETS protein Elf-1

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Abstract Elf-1 is a lymphoid-specific transcription factor that belongs to the ETS protein family. It can bind to DNA target sequences within a variety of cytokine genes. We demonstrate that Elf-1 is constitutively localized in the nucleus which is dependent on the presence of amino acids 86–265. Analysis of Gal4-Elf-1 fusion proteins revealed that the N-terminal 86 amino acids of Elf-1 contain a transcriptional activation domain, the activity of which is attenuated by an internal repression domain. Furthermore, Elf-1 interacts specifically with the E74 target sequence and can stimulate transcription driven by the E74 site independent of mitogenic signaling. Thus, Elf-1 is able to stimulate gene transcription which may be required for the development and activity of lymphocytes.

Key words: Elf-1; ETS protein; Gene expression; Lymphocyte; Nuclear localization; Transactivation

1. Introduction

The DNA binding protein Elf-1 is a member of the ETS protein family which is involved in developmental processes [1,2], mitogenic activation of cells [3], oncogenesis [4,5] and viral gene activation [6,7]. All ETS proteins require a $GGA^{A}/_{T}$ core sequence for specific DNA binding whereby flanking nucleotides determine the specificity and affinity of a single ETS protein towards a particular binding site [8,9]. Since almost always several ETS proteins can potentially bind to a given target sequence, preferential binding of a unique ETS protein can be achieved by selective interaction with another transcription factor bound to a flanking DNA sequence, or by tissue-specific expression [10]. Furthermore, the activity of some ETS proteins can be regulated at the level of phosphorylation as was shown, for example, for the ETS proteins Ets-1, Ets-2, Elk-1 and Sap-1a [3,11].

Elf-1 was described as a lymphoid-specific ETS protein [12] that can bind autonomously to different promoter sequences of lymphoid-specifically expressed genes, including those encoding interleukin-3 [13], granulocyte-macrophage colony-stimulating factor [14], CD4 [15] and interleukin-2 (IL-2) receptor α [16], as well as to the long terminal repeats of T-cell trophic viruses like HIV-2 [17] and HTLV-I [18]. Elf-1 had also been suggested to be a component of the NF-AT transcription factor complex which mediates the cyclosporine A-sensitive activation of the *IL-2* gene [12], because Elf-1 could potentially bind within the NF-AT site of the IL-2 promoter. However, cloning of *NF-AT* genes revealed that NF-AT does not contain Elf-1 nor does NF-AT require any ETS protein to bind to a site with a GGAA core sequence [19,20]. Furthermore, Elf-1 binds with high affinity to the immunoglobulin heavy-chain enhancer π site [21] and to the promoters of the tyrosine kinase encoding genes *blk* and *lyn* [22] and may thus contribute to the proper function of B-cells.

In this report we demonstrate that the Elf-1 protein is constitutively localized within the cell nucleus. Also, we identify an N-terminal activation domain as well as a central negative regulatory domain and show that Elf-1 mediates transcription from the E74 binding site independent of extracellular signals funneling through mitogen-activated protein kinases (MAPK).

2. Experimental procedures

2.1. DNA cloning

Segments of Elf-1 cDNA were cloned into the pABGal-linker plasmid to generate eukaryotic expression vectors for the different Gal4-fusions or into a CMV enhancer/promoter-driven mammalian expression vector providing a hemagglutinin (HA) tag [23]. The *luciferase* reporter gene constructs tk80-luc, $E74_3$-tk80-luc and $Gal4_2$-tk80-luc have been described before [24].

2.2. Expression of proteins

Rabbit kidney epithelial-like cells (RK13) were cultured in RPMI+10% FCS. 30 μg plasmid DNA were used to transfect 50%-confluent RK13 cells on a 10 cm dish using the calcium-phosphate coprecipitation method. After an incubation period of 8 h the cells were rinsed twice in 10 mM Hepes, pH 7.3, 142 mM NaCl, 6.7 mM KCl and grown for additional 12–18 h in RPMI+10% FCS. Then, the cells were washed with PBS and were taken off the dish with 40 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA. After centrifugation (3 min, 1000×*g*) the cell pellet was taken up for lysis in 0.5 ml of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 10 mM NaF, 0.5% Triton X-100, 0.05% sodium deoxycholate, 1 mM DTT, 0.5 mM PMSF. After 20 min on ice cell debris was removed by centrifugation (10 min, 10 000×*g*) and the protein extract was frozen in liquid nitrogen and kept at −80°C.

2.3. Luciferase assays

RK13 cells were transiently transfected and lysed 36 h post-transfection [24]. The cell lysate was used to measure the luciferase activity in a buffer containing 25 mM glycylglycine, 15 mM $MgSO_4$, 5 mM ATP and 0.1 mM D-luciferin (AppliChem). As a control for transfection efficiency, a β-galactosidase expression vector was always co-transfected and β-galactosidase activity was determined by incubating 50 μl cell lysate with 1 mg/ml *o*-nitrophenyl β-D-galactopyranoside (AppliChem) in 60 mM Na_2HPO_4, 39 mM NaH_2PO_4, 10 mM $MgSO_4$, 2 mM DTT. The generated *o*-nitrophenolate was measured at 405 nm in a spectrophotometer.

2.4. Immunofluorescence studies

HeLa cells growing on cover-slips were transfected using the calci-

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Abbreviations: HA, hemagglutinin; IL-2, interleukin-2; MAPK, mitogen-activated protein kinase; RK13, rabbit kidney epithelial-like cells

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um phosphate coprecipitation method [24]. After 24 h the cells were washed twice with PBS before fixing them for 6 min in methanol at −20°C. The cells were washed 3 times with PBS and incubated for 20 min with PBS/1% bovine serum albumin prior to a 1 h incubation with a murine monoclonal antibody against the HA epitope [23]. After a further washing of the cells an incubation with goat α-mouse antibodies coupled with fluorescein isothiocyanate was performed. The cells were washed twice with PBS, once in PBS containing 1 μg/ml Hoechst dye 33258, twice with PBS and once with water. After mounting on a slide with Entellan (Merck), indirect immunofluorescence microscopy was performed with a 100× lens and a 10× ocular.

2.5. Gel retardation assay

Protein extract (2 μ)l was incubated with 0.1 μl of ^{32}P-labeled oligonucleotide (0.5–1 ng) and 8 μl of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.05% non-fat dry milk, 5% glycerol, 0.1 μg/μl poly(dldC)(dldC), 10 mM DTT. The reaction was performed for 30 min at room temperature. Before electrophoresis on a 5% acrylamide gel (0.5×TBE) 2 μl of 10% Ficoll 400, 15% glycerol, 40 mM EDTA were added to the binding reactions. After electrophoresis at room temperature the gel was dried and exposed to an X-ray film for 12 h. The sequence of the E74-oligonucleotide was (asterisks highlight guanine residues mutated to cytosine in the mE74-oligonucleotide): 5′-AGCTTCTCTAGCTGAATAACCG*G*AAGTAACTCATCGTCG-A-3′.

3. Results

3.1. Intracellular localization of Elf-1

Several ETS proteins have been proven to stimulate, or even repress, gene transcription [3,8,9]. By necessity, a transcription factor has to act in the nucleus. But some transcription factors reside in the cytoplasm and require signal-induced translocation from the cytoplasm to the nucleus like NF-AT [25]. In order to investigate the intracellular localization of Elf-1 and map its nuclear translocation region by indirect immunofluorescence microscopy, HeLa cells were transiently transfected with Elf-1 expression vectors. Since all expressed Elf-1 proteins possessed an N-terminal HA-tag, a monoclonal antibody directed against the HA-tag was employed for detection.

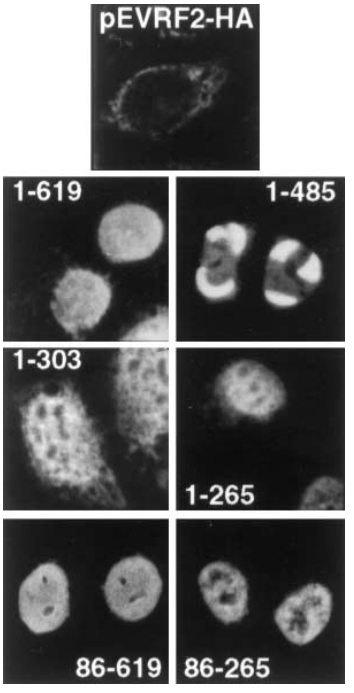

Fig. 1. The nuclear localization of Elf-1 was assayed by indirect immunofluorescence microscopy. HeLa cells were transiently transfected with full-length HA-tagged Elf-1 (1–619), respective truncations or the empty expression vector pEVRF2-HA and subsequently stained with a monoclonal antibody directed against the HA-epitope.

As a control, we stained HeLa cells transfected with the empty expression vector (pEVRF2-HA) which gives rise to a short peptide containing the HA-epitope. As demonstrated before [26], this peptide was localized within the cytoplasm (Fig. 1). Analysis of the full-length Elf-1 protein (1–619) showed that it was exclusively localized within the cell nucleus (Fig. 1). An identical result was obtained with untagged Elf-1 stained with an antibody directed against the C-terminal 19 amino acids (data not shown). Similarly, the C-terminal truncations Elf1-485 and Elf1-265 gave a nuclear staining, whereas a whole-cell staining was unexpectedly observed with Elf1-303. Deletion of the N-terminal 85 amino acids did not affect the nuclear localization, as shown with Elf86-619 and Elf86-285. Interestingly, the staining pattern of Elf1-485 revealed an uneven distribution of this protein in the nucleus which was reproducibly observed.

The presented data were obtained with continuously growing HeLa cells cultivated in medium containing 10% fetal calf serum. We have also analysed serum-starved HeLa cells before or after mitogenic induction with phorbol-ester, but no difference in the staining pattern of Elf-1 was noticeable (data not shown). We therefore conclude that Elf-1 is a constitutive nuclear protein.

3.2. Mapping of a transactivation domain

To study the transcriptional potential of Elf-1, we fused parts of Elf-1 to the DNA binding-domain of the yeast protein Gal4 (Fig. 2). A Western blot revealed that all Gal4-Elf-1 fusion proteins were expressed to comparable levels (not shown). Then, these fusion proteins were tested with a reporter construct driven by two Gal4 binding sites. While the Gal4 moiety itself was transcriptionally inert (see Gal4 in Fig. 2), the Gal4-Elf-1 fusion (G/E1-619) raised the relative luciferase activity by a factor of 10. This effect was binding-site specific, since a luciferase reporter devoid of the Gal4 binding-sites (tk80-luc) was not responsive to G/E1-619 (Fig. 2, right panel). Thus, Elf-1 has the potential to activate transcription.

Deletion of C-terminal amino acids down to position 265 did not alter the transactivation potential, as demonstrated with the G/E1-485, G/E1-303 and G/E1-265 fusion proteins (Fig. 2). Surprisingly, further deleting amino acids from the C-terminus raised transcriptional levels with the G/E1-205, G/E1-104 and G/E1-86 constructs, indicating that amino acids 206-265 may contain an inhibitory domain. Furthermore, deletion of the first 85 amino acids resulted in transcriptionally inactive proteins (see G/E86-265 and G/E86-202). Altogether, these results suggest that the N-terminal 86 amino acids of Elf-1 encompass a transactivation domain and that amino acids 206–265 attenuate the transactivation potential of the N-terminal activation domain.

3.3. E74 site dependent transcription mediated by Elf-1

The ETS domain of Elf-1 is highly homologous to that of the Drosophila E74 protein, which resulted in the name **E74-l**ike-**f**actor [12], although otherwise the two proteins are different. Thus, we tested whether Elf-1 could bind to the E74 target sequence. As shown in a gel retardation assay (Fig. 3)

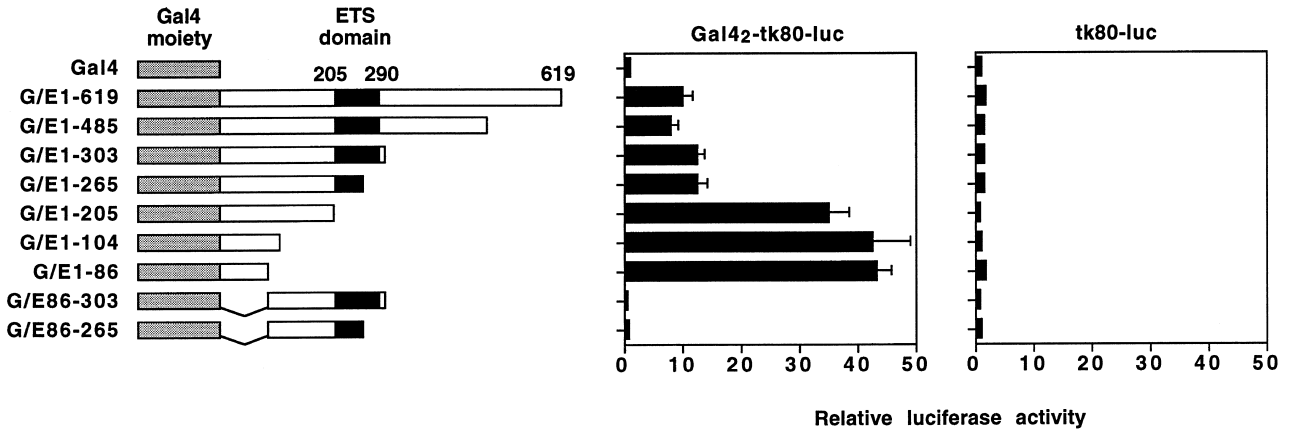

Fig. 2. Mapping of the Elf-1 transactivation domain. Transcriptional analysis of the depicted Gal4-Elf-1 fusion proteins was measured in RK13 cells using a Gal4 binding site-driven luciferase reporter construct (Gal4$_2$-tk80-luc) or a reporter construct devoid of the Gal4 binding site (tk80-luc).

Elf-1 can bind to the E74 target sequence. In order to assess the specificity of this DNA binding, competition experiments with unlabeled oligonucleotides were performed. While a 100-fold molar excess of unlabeled E74-oligonucleotide completely abolished Elf-1 interaction with the ^{32}P-labeled E74-oligonucleotide (Fig. 3), no effect was observable with the mE74-oligonucleotide in which the GGAA core sequence of the E74 binding site was changed to CCAA. Similarly, a different ETS binding site (GABP), to which the GABP protein complex binds with high affinity [8,9], was incapable to compete with the ^{32}P-labeled E74-oligonucleotide for binding to Elf-1. These results indicate that Elf-1 interacts specifically with the E74 binding site.

To study the transcriptional capacity of Elf-1 and its derivatives, an E74 binding site-driven luciferase reporter construct was used in transient transfection experiments. As a control we analyzed the ETS protein Elk-1 that is not active without mitogenic stimulation of cells [3]. In this functional assay, Elf-1 as well as the Elf1-485 and Elf1-303 truncations were approximately 7-fold more active than Elk-1 (Fig. 4A). No activation of transcription was noticed with the tk80-luc reporter being devoid of E74 binding sites (Fig. 4A), demonstrating that Elf-1 acts site specifically. Further, a control gel retardation assay showed that comparable amounts of Elk-1 and the different Elf-1 constructs were produced in RK13 cells (Fig. 4B). Deleting the first 85 amino acids decreased the transactivation potential (see Elf86-619, Elf86-485 and Elf86-303 in Fig. 4A) confirming that amino acids 1–86 contain a transactivation domain. However, the residual activity of the N-terminally deleted Elf-1 proteins implies that amino acids 86–303, when bound to DNA via the ETS domain, might still slightly stimulate transcription. Please note that we could not test the impact of amino acids 206–265 on the activation potential with the E74 site-driven luciferase reporter, since these amino acids are part of the DNA binding domain and thus prohibit their deletion. Furthermore, in contrast to Elk-1, we could not find a significant increase of the activation potential of Elf-1 after activation of MAPKs (Fig. 4C) or upon stimulation with phorbol esters (not shown), indicating that mitogenic signaling does not regulate Elf-1 function.

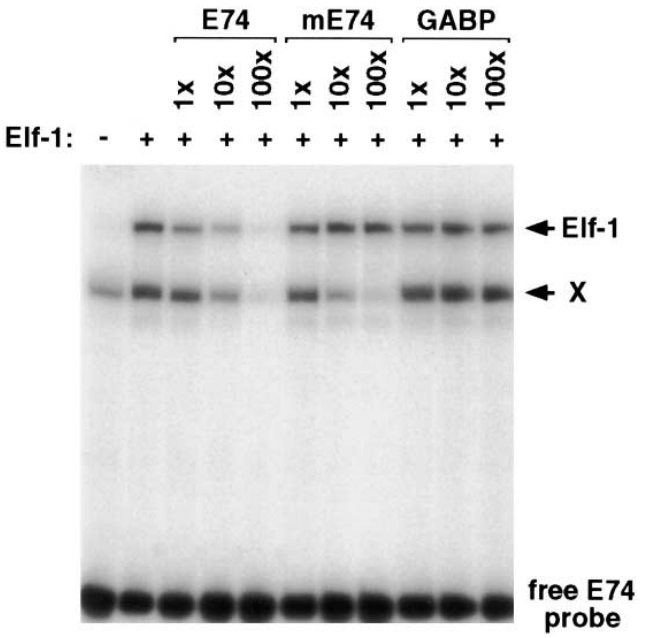

Fig. 3. Interaction of Elf-1 with the E74 binding site. In vitro translated Elf-1 was employed in a gel retardation assay with the ^{32}P-labeled E74-oligonucleotide. The specificity of binding activity was assessed with different molar excesses of the indicated non-radioactive competitor oligonucleotides. 'X' denotes an unidentified complex not containing Elf-1.

4. Discussion

In this study, we have provided evidence that the transcription factor Elf-1 is constitutively localized within the cell nucleus. Since Elf-1, with a molecular weight of 68 kDa, is too big for a passive transport through the nuclear pores, active transport has to be involved in the nuclear localization of Elf-1. The first 85 amino acids of Elf-1 are of no relevance for the intracellular localization; similarly, deleting C-terminal amino acids down to position 485 had no impact on the nuclear localization of Elf-1, and even Elf1-265, or Elf86-265, was localized within the cell nucleus. This suggests that a nuclear localization signal is situated within amino acids 86–265. Surprisingly, Elf1-303 was present in both the cytoplasm and the nucleus. This might be due to the fact that amino acids 266–303, in the context of Elf1-303 but not in the context of full-

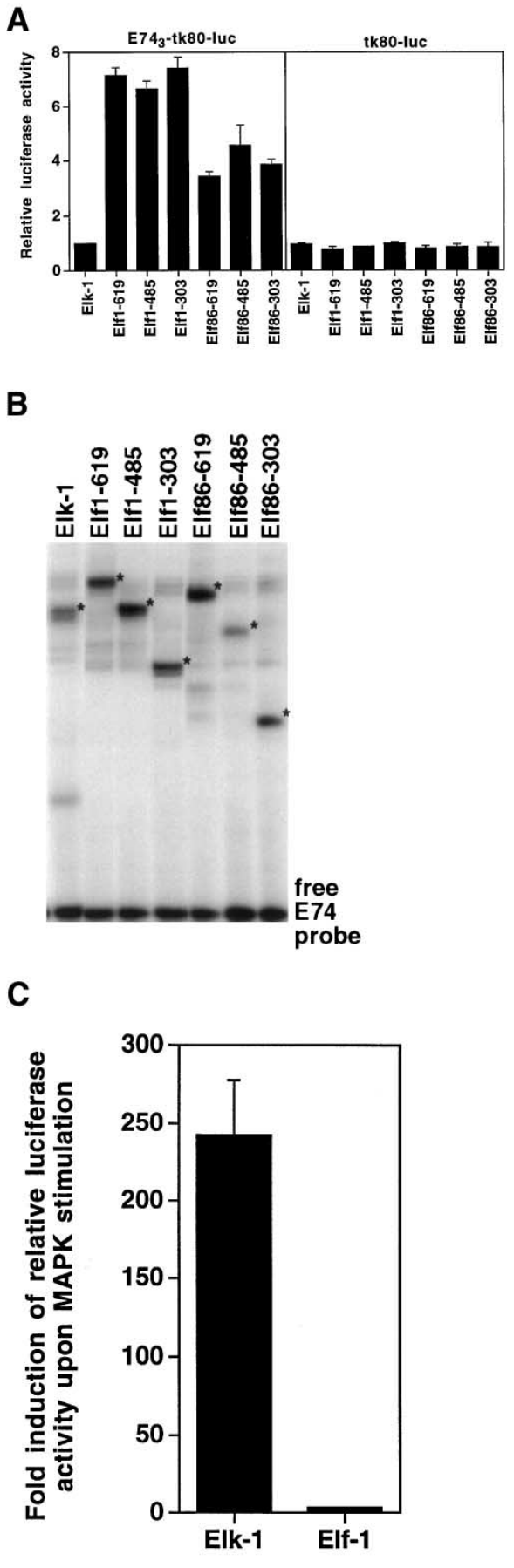

Fig. 4. Elf-1 mediated stimulation of E74 binding site-driven transcription. A: Elf-1 transcriptional activity was studied in transient transfections of RK13 cells using an E74 binding site-driven luciferase reporter construct (E74$_3$-tk80-luc) or, as a control, the tk80-luc reporter. B: Whole-cell extracts of transiently transfected RK13 cells were employed in a gel retardation assay with ^{32}P-labeled E74-oligonucleotide. Asterisks mark the complexes containing the respective indicated protein. C: Transcriptional activation upon MAPK stimulation. RK13 cells were cotransfected with p44 MAPK, an upstream activator of MAPK (constitutively active Raf-1 kinase [24]) and either Elk-1 or Elf-1.
←

length Elf-1 or Elf1-485, may mask a nuclear translocation signal within amino acids 86–265.

A transactivation domain of Elf-1 was mapped to the N-terminal 86 amino acids. Interestingly, deletion of amino acids 206–619 resulted in an increase of the transactivation potential. Thus, the C-terminal amino acids may impede transactivation by Elf-1. Specifically, amino acids 206–265 may be responsible for this intramolecular inhibition of transactivation, since deletion of amino acids downstream of position 266 had no impact on the function of Gal4-Elf-1 fusions. However, our results were obtained with Gal4-fusion proteins which may blur the real situation. In this regard, it has been shown for Sap-1a that the ETS domain, when not bound to DNA as in a Gal4-fusion at a Gal4 binding site-containing promoter, abrogates transcriptional activity of the Sap-1a protein [26]. Here, we have similarly observed that the complete deletion of the ETS domain in the G/E1-205 truncation is required for maximal transactivation, suggesting again that the ETS domain may negatively affect transactivation.

The binding of Elf-1 to different promoters of lymphoid-specific genes was described [13–17], but a direct impact of this factor for transcriptional activity of these promoters was not shown. Here, we demonstrate that Elf-1, when bound to the E74 target sequence, may stimulate transcription independent of mitogenic signaling. These results are in agreement with two recent reports demonstrating that Elf-1 activates transcription of the *immunoglobulin* heavy-chain gene and the B-cell-specific protein kinase genes *blk* and *lyn*, the latter also being expressed in monocytes [21,22]. Therefore, Elf-1 activity may be essential for the function of B-cells. In addition, Elf-1 may contribute to the regulation of the terminal *deoxynucleotidyltransferase* gene which is required during early B- and T-cell development [27]. However, since this gene is shut down in mature lymphocytes where Elf-1 is expressed [22], Elf-1 cannot be the sole regulator of the terminal *deoxynucleotidyltransferase* gene. Rather, as the analysis of lymphoid-specifically expressed genes such as IL-2 has often implicated multi-protein complexes in the regulation of gene activity, Elf-1 may be just one factor out of many that is required but not sufficient for the control of promoter activity.

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