digest libraries. Therefore, a possible concern is that the cloning efficiency in the random shear approach may be too low to be practical and that large amounts of target DNA would be needed to produce multiple representation fosmid libraries. However, results obtained in the construction of the *N. punctiforme* library clearly demonstrate that 30- to 40-kb fragments produced by random shearing (followed by end repair and dephosphorylation) can be cloned into pFOS-LA at high efficiency (>10^5 cfu/µg target DNA). This level of cloning efficiency is clearly adequate for producing genomic libraries of high statistical representation from microgram and submicrogram quantities of DNA. The cloning efficiency observed in these experiments, taken together with a tolerance for smaller (even slightly degraded) starting DNA, enables and encourages the use of pFOS-LA for making libraries from small amounts of DNA obtained from animal and plant tissues, microbial material, and flow-sorted chromosomes.

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Address correspondence to Jonathan L. Longmire, Mail Stop M888, Los Alamos National Laboratory, Los Alamos, NM, USA. e-mail: longmire@telomere.lanl.gov

ELAM-1/E-selectin promoter contains an inducible AP-1/CREB site and is not NF-κB-specific

Liselotte E. Jensen and Alexander S. Whitehead
University of Pennsylvania School of Medicine, Philadelphia, PA, USA

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The innate immune system plays an important role as a first defense against pathogens and involves the recognition of bacteria and viruses, and byproducts thereof, by Toll receptors on immune-competent cells (1). Activated cells synthesize and secrete cytokines, which in turn activate systemic responses directed at clearing the pathogen. Two important cytokines are interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). These cytokines, as well as the Toll receptors, initiate intracellular signaling cascades that activate nuclear factor κB (NF-κB), activator protein-1 (AP-1), and cAMP responsive element binding proteins (CREBs), which are transcription factors essential for the regulation of numerous genes, many of which play important roles in immunological processes. Subunits of the AP-1 and the CREB protein families may form both homo- and heterodimers; the latter may comprise subunits both from within and between protein families. The signaling pathways leading to activation of at least NF-κB and AP-1 are common proximal to the receptors but diverge downstream to provide specificity (2). A common tool for examining signaling cascades is the luciferase (or chloramphenicol acetyltransferase) reporter assay. Such assays offer advantages such as simple protocols, no requirement for highly efficient transfections, and the possibility of elucidating molecular orders of signaling factors and determining branch-points of diverging signaling cascades.

The endothelial leukocyte-adhesion molecule (ELAM)-1 (also called E-selectin) promoter contains three NF-κB sites, two of which are partially overlapping, that have been reported to be required for full induction by cytokines. A putative AP-1 site at position -499 to -493 within the promoter has been shown not to affect cytokine induction (3). Consequently, this promoter is often used to drive expression of luciferase in reporter assays and is frequently considered to be an "NF-κB-specific" promot-

Table 1. Sequences of Wild-Type and Mutant Transcription Factor Binding Sites

No.	Site	Position	Sequence	Mutant Sequence
2	NF-κB.1/2	-126 to -107	5′-GGATATTCCC GGGAAAGTTT-3′	5′-GGATATTGGC GGGAAAGTTT-3′
3	NF-κB.3	-94 to -85	5′-GGGGATTTCC-3′	5′-GGCGATTTCC-3′
4	NF-κB.1/2 + NF-κB.3	-126 to -107 -94 to -85	5′-GGATATTCCC GGGAAAGTTT-3′ 5′-GGGGATTTCC-3′	5′-GGATATTGGC GGGAAAGTTT-3′ 5′-GGCGATTTCC-3′
5	AP-1.1	-499 to -493	5′-TGAGTCA-3′	5′-CAAGTCA-3′
6	AP-1.2	-153 to -146	5′-TGACATCA-3′	AP1: 5′-TGACATTG-3′
7	AP-1.2	-153 to -146	5′-TGACATCA-3′	CREB: 5′-TGTGATCA-3′

Numbers to the left identify the constructs depicted in Figure 1 and described in the text.

er. We searched the -729 to +52 region of the ELAM-1 promoter against the TRANSFAC v4.0 database available via the Baylor College of Medicine HGSC search launcher at http://searchlauncher.bcm.tmc.edu. The previously described NF-κB and AP-1 sites were readily identified (NF-κB.1/2, NF-κB.3, and AP1.1) (Figure 1). However, an additional AP-1/CREB binding site was found immediately upstream of the NF-κB sites (AP1.2) (Figure 1). An extensive literature search revealed that this "second" AP-1 site had previously been shown to bind both CREB and AP-1 subunits and to be involved in induction by cytokines (4–7). The binding of CREB has been shown to down-regulate transcription in response to TNF-α (6,7). It has further been suggested that AP-1 may interact directly with NF-κB and thereby regulate cytokine-induced transcription (5).

Given the apparent discrepancy between these data and the use of the ELAM-1 promoter as a NF-κB-specific promoter, we wished to determine if the AP-1 sites could influence cytokine-driven transcription from this promoter. The -729 to +52 region of the ELAM-1 gene (112–892 in GenBank® accession no. M64485) was amplified using primers containing appropriate enzyme restriction sites (5′-GGTACCGAGCTCCCAAAGTGGTGGG-3′, *Sac*I site underlined; and 5′-TTACTTAGATCTGTCTCAGGTCAG-3′, *Bgl*II site underlined) to facilitate directional cloning into the pGL2-Basic promoterless luciferase-reporter construct (Promega, Madison, WI, USA). The NF-κB and AP-1 sites were mutated into nonfunctional/nonbinding sites (Figure 1 and Table 1) using site-directed mutagenesis (QuikChange™; Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Each individual luciferase reporter construct was co-transfected into HepG2 cells with a Renilla luciferase reporter construct (pRL-null; Promega) that was used to standardize for transfection efficiencies. Cells were treated with medium only, 10 ng/mL IL-1 (National Cancer Institute, Frederick, MD, USA) or 50 ng/mL TNF-α (Zeneca Pharmaceuticals, Macclesfield, UK). Cell lysates were harvested after 4 h, and luciferase and Renilla luciferase activities were determined (Dual-Luciferase Reporter Assay System; Promega) according to the manufacturer's instructions. Mutagenesis of any of the transcription factor binding sites resulted in approximately 50% reduced constitutive activity of the mutant promoters (constructs 2–7 compared to construct 1 in Figure 1). This indicates that low levels of constitutively active NF-κB and AP-1 are present in the cells. In response to IL-1 and TNF-α, the wild-type promoter (Figure 1, construct 1) resulted in 290-fold and 51-fold increases in lu-

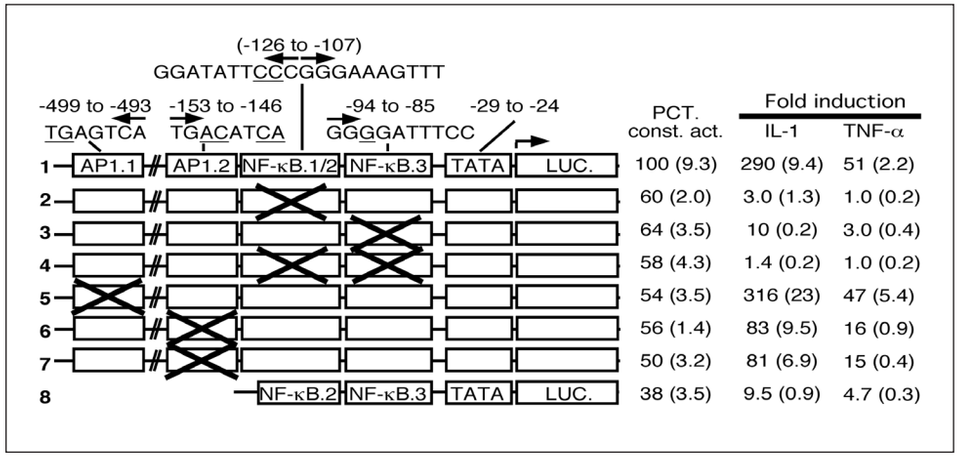

Figure 1. Functional activity of transcription factor binding sites in the ELAM-1 promoter. Positions of transcription factor binding sites, together with sequences, and the TATA box are schematically represented (not drawn to scale) at the top. Arrows indicate "direction" of binding sites. Underlined bases are those that were mutated to eliminate transcription factor binding (see also Table 1). The bent arrow indicates transcription start site for expression of the luciferase coding sequence (LUC.). Numbers to the left identify the constructs. Constructs were co-transfected with pRL-null into cells. Cells were treated with medium only, IL-1, or TNF-α, and luciferase and Renilla luciferase activities were determined after 4 h. Luciferase values were standardized against Renilla luciferase. Numbers to the right indicate activities of individual promoters. The constitutive activities (PCT. const. act.) of individual mutant ELAM-1 promoters are expressed as percentages of the activity of the wild-type promoter (defined as 100%). Responses to IL-1 and TNF-α are expressed as fold induction of promoter activity compared to that in cells treated with medium only. Triplicate samples were analyzed, and standard deviations are indicated in parentheses. Results are representative of three independent experiments.

ciferase activity, respectively. Introduction of mutations within the two overlapping NF-κB sites (NF-κB.1/2, construct 2) or the third NF-κB site (NF-κB.3, construct 3) resulted in dramatically lower cytokine-driven promoter induction. The cytokine response was completely abolished when all NF-κB sites were targeted (construct 4). In contrast, mutagenesis of the most upstream AP-1 site (AP1.1, construct 5) had no effect on cytokine induction (Figure 1). Since the second AP-1 site may bind both AP-1 and CREB, two different mutant constructs were generated to target this site. In construct 5, bases often targeted for examination of AP-1 binding were mutated; in construct 6, bases considered essential for CREB binding were mutated. The cytokine-driven expression of luciferase from these constructs were remarkably similar and, most significantly, were lower in terms of fold induction compared to that of the wild-type construct [i.e., the wild-type (construct 1) response to IL-1 and TNF-α was approximately 3.5 times greater than that of either construct 6 or 7 (Figure 1)]. This clearly demonstrates that the second AP-1/CREB site (AP1.2) is involved in transcriptional activation and confirms previous reports (4,5) indicating the involvement of this transcription factor binding site in cytokine-driven induction. The AP-1.2 site on its own, in the absence of any NF-κB sites, is unable to facilitate a cytokine response, as evidenced by the lack of enhanced transcription from construct 4. However, given that we showed earlier that there is constitutive NF-κB activity in cells, the AP1.2 site may result in increased transcription in response to cytokines even in the absence of increased NF-κB activation. Clearly, data derived from experiments using this promoter should be interpreted with great caution, since the promoter cannot be considered to be truly specific for NF-κB.

To evaluate whether a shorter segment of the ELAM-1 promoter could be used in NF-κB-specific reporter assays instead of the full-length promoter, the region spanning from the *Sac*I cloning site to the *Sma*I site within the NF-κB.1/2 site was deleted to generate a minimal promoter spanning -116 to +52 (construct 8). Construct 1 was cut with *Sac*I and *Sma*I, and the vector fragment was gel-purified. The *Sac*I overhanging end was removed, and the vector was religated. The resulting construct was co-transfected into cells with the Renilla luciferase reporter and cells subsequently treated with medium only, or IL-1 or TNF-α. Although this minimal ELAM-1 promoter construct (construct 8) had a lower capacity to respond to either cytokine (Figure 1) than the construct encoding the full-length promoter, significant increases in luciferase activities, sufficient for most experimental protocols, were observed (9.5-fold increase in response to IL-1 and 4.7-fold for TNF-α).

Reporter constructs that contain artificial minimal promoters with multiple copies of a NF-κB consensus site placed upstream of the luciferase coding region can also be used for NF-κB-specific reporter assays instead of reporter constructs containing the full-length ELAM-1 promoter. Such constructs are commercially available from several sources [e.g., Stratagene and BD Biosciences Clontech (Palo Alto, CA, USA)]. We tested the capacity of the pNF-κB-Luc (Stratagene) reporter construct, which contains five repeated NF-κB binding sites, to respond to cytokines. In response to IL-1 and TNF-α, 12-fold (SD = 0.3) and 5.3-fold (SD = 0.2) increases in expression were observed, respectively. These fold increases are very similar to those of construct 8 containing the minimal ELAM-1 promoter. Constitutive expression from pNF-κB-Luc was 759 (SD = 32) times higher than that from construct 1 containing the full-length ELAM-1 promoter. Therefore, assays in which construct 1 is replaced with pNF-κB-Luc will require careful adjustment of the absolute amount of reporter construct transfected into cells to ensure that luciferase readouts remain within the linear range of the luminescence detection instrument used.

The above data demonstrate that while the full-length ELAM-1 promoter may be used in reporter assays for general stress responses, it cannot be considered to be NF-κB-specific. A more appropriate alternative for specifically measuring NF-κB activity is a minimal ELAM-1 promoter that does not contain any of the AP-1/CREB transcription factor binding sites described here. Such a minimal promoter can easily be derived from existing constructs, and the transition from using the full-length promoter to using the minimal promoter involves little optimization because levels of constitutive expression are relatively similar.

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Address correspondence to Alexander S. Whitehead, Department of Pharmacology and Center for Pharmacogenetics, University of Pennsylvania School of Medicine, 153 Johnson Pavilion, 3620 Hamilton Walk, Philadelphia, PA 19104-6084, USA. e-mail: aswhitehead@pharm.med.upenn.edu