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Three NF-κB Binding Sites in the Human E-Selectin Gene Required for Maximal Tumor Necrosis Factor Alpha-Induced Expression

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Transcription of the gene encoding the endothelial cell-leukocyte adhesion molecule (ELAM-1; E-selectin) is induced in response to various cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1. A DNase I-hypersensitive site in the 5′ proximal promoter region of the E-selectin gene is observed in human umbilical vein endothelial cells only following TNF-α treatment, suggesting the presence of a TNF-α-inducible element close to the transcriptional start site. Transient transfection studies in endothelial cells demonstrated that 170 bp of upstream sequences is sufficient to confer TNF-α inducibility. Systematic site-directed mutagenesis of this region revealed two regulatory elements (−129 to −110 and −99 to −80) that are essential for maximal promoter activity following cytokine treatment. Protein binding studies with crude nuclear extracts and recombinant proteins revealed that the two elements correspond to three NF-κB binding sites (site 1, −126; site 2, −116; and site 3, −94). All three sites can be bound by NF-κB when used as independent oligonucleotides in mobility shift assays. However, within the context of a larger promoter fragment, sites 2 and 3 are preferentially occupied over site 1. These data are consistent with results obtained in transfection studies demonstrating that mutations in sites 2 and 3 are more detrimental than mutations within site 1. Hence, inducibility of the E-selectin gene requires the interaction of NF-κB proteins bound to multiple regulatory elements.

The endothelial cell-leukocyte adhesion molecule (ELAM-1; E-selectin), a member of the selectin family of cell surface glycoproteins, mediates the adhesion of leukocytes to the endothelium and is thought to play a central role in the recruitment of circulating blood cells to sites of inflammation (for a review, see reference 7). E-selectin expression is restricted to venular and capillary endothelial cells and is observed only at sites of acute and chronic inflammation (10, 30). Cultured human umbilical vein endothelial cells (HUVE cells) express E-selectin on the cell surface only when exposed to inflammatory cytokines such as tumor necrosis factor-α (TNF-α) or interleukin-1 (IL-1). Induction of E-selectin expression in HUVE cells is rapid and transient, with mRNA levels peaking 4 h posttreatment and returning to basal levels by 24 h (5). Nuclear run-on experiments have demonstrated that the regulation of E-selectin expression is mediated at the transcriptional level (14, 27, 38).

TNF-α and IL-1 are both known to be potent activators of nuclear factor kappa B (NF-κB). NF-κB is a ubiquitous transcription factor implicated in the activation of numerous cellular and viral genes in response to a wide variety of stimuli (for reviews, see references 1, 6, and 16). NF-κB activity is mediated by homo- or heterodimeric combinations of distinct proteins that belong to the Rel family of transcription factors and share a related DNA binding and dimerization domain (the Rel domain). The five NF-κB subunits that have been characterized to date include p50/p105, p52/p100, p65 (RelA), c-REL, and Rel B (for a review, see reference 24). Individual homo- and heterodimeric combinations of these subunits exhibit unique DNA-binding specificities and transcriptional activation properties (23, 33). In most cell types, including endothelial cells, NF-κB is found in an inactive form in the cytoplasm associated with the inhibitor molecule IκB (for reviews, see references 3 and 15). Upon stimulation, NF-κB is released from IκB and enters the nucleus to activate transcription.

Sequence analysis of the E-selectin proximal promoter region has revealed the presence of an NF-κB binding site located between 94 and 85 bp upstream from the transcriptional start site. Mutations within this site block IL-1-induced gene expression (38). Although this NF-κB binding site is necessary for cytokine induction in the context of the E-selectin promoter, a single copy of this site does not confer IL-1-induced gene expression when it is fused to a heterologous promoter. Two additional regulatory elements have been identified in the E-selectin gene on the basis of in vitro DNA-binding studies using nuclear extracts from HUVE cells. The two binding activities have been termed NF-ELAM1 and NF-ELAM2 (19). Mutations of these protein-binding sites reduce IL-1-induced expression of the E-selectin gene. However, neither of the two sites is sufficient to confer IL-1 inducibility (19). Recent studies have revealed that the NF-ELAM1 site is recognized by members of the ATF family of transcription factors (18), including ATFα, ATF2, and ATF3. With recombinant proteins, it was shown that all three proteins, ATFα, ATF2, and ATF3, physically associate with NF-κB in vitro (22). These interactions may contribute to the enhanced, cytokine-mediated activation observed when the NF-ELAM1 site is linked to the NF-κB site (19, 22). The role of the NF-ELAM2-binding site in cytokine-induced expression of E-selectin and the identity of the protein(s) that interact with this site have not yet been established.

The three regulatory DNA motifs in the E-selectin promoter were identified by their ability to be recognized by distinct nuclear DNA-binding activities (NF-κB, NF-ELAM1, and NF-ELAM2, respectively). To obtain a more complete understanding of the mechanisms underlying cytokine-induced expression of the E-selectin gene in endothelial cells, we con-

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ducted a systematic mutational analysis of the entire proximal promoter region. Our studies have identified a new *cis*-regulatory element of the E-selectin promoter that is essential for cytokine-induced expression. Binding studies with nuclear extracts and recombinant proteins demonstrated that this region contains two closely spaced NF-κB binding sites. Transfection studies using wild-type and mutant promoter templates established that occupancy of both sites, in addition to the previously identified NF-κB site, is essential for maximal promoter activity. Hence, cytokine induction of the E-selectin gene in endothelial cells involves an interplay between NF-κB molecules bound to multiple sites within the promoter.

MATERIALS AND METHODS

Cell culture and transfections. HUVE cells were obtained from Clonetics and grown in endothelial growth medium (EGM; Clonetics) on Primaria dishes (Falcon). Cells from passage four were used for all transfection studies. Cells were plated into six-well Primaria culture dishes (7×10^4 cells per well) 24 h prior to transfection. Transfections were performed with Lipofectin (Gibco-BRL) according to the manufacturer's recommendations. Briefly, reporter DNA (10 μg) was mixed with 2 μl of lipofectin and 188 μl of serum-free medium (Opti-MEM; Gibco-BRL). After a 30-min incubation, Opti-MEM (800 μl) was added, and the mixture was applied onto the cells that had been washed twice with Opti-MEM. Four hours later, the medium was changed to EGM and the cells were harvested 48 h after transfection. Recombinant TNF-α was used at a concentration of 20 ng/ml for 4 h before harvesting the cells. Cells were lysed in 150 μl of lysis buffer (Promega), and 10 μl of the extract was used for the luciferase assay (Promega). Extracts were quantitated for protein concentration by the Bradford method (Bio-Rad).

DNase I hypersensitivity mapping. Nuclei were isolated from untreated HUVE cells or from HUVE cells (passage six) that had been treated with TNF-α (20 ng/ml) for 4 h before harvesting. Nuclei were prepared as described elsewhere (13). After mild DNase I treatment with the amounts indicated in the legend to Fig. 1, the reaction mixture were treated with proteinase K overnight (13). The DNA was purified by phenol-chloroform extraction and was restricted with *Eco*RI. DNA fragments were separated on a 0.8% agarose gel and transferred to nitrocellulose (Schleicher & Schuell). Prehybridization and hybridization to the random-primed ^{32}P-labeled *Stu*I-*Eco*RI fragment derived from the genomic clone TPU19 (34a) were carried out at high stringency (42°C; 50% formamide–5× SSPE [1× SSPE is 0.15 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA, pH 7.7]–10× Denhardt's–0.5% sodium dodecyl sulfate (SDS)–0.1 mg ml^{-1} salmon sperm DNA). Filters were washed twice with 0.2× SSPE at 68°C for 30 min and were exposed to Kodak X-Omat AR film.

Plasmid constructs. The wild-type fragment (−730 to +52) of the E-selectin promoter was generated by PCR using two gene-specific primers and the genomic clone TPU19 (unpublished data) as the template. The two primers carried base pair substitutions that introduced a *Sac*I site at the 5′ end of the fragment (5′ *Sac*I primer) and a *Bgl*II site at the 3′ end (3′ *Bgl*II primer; see below). The resulting fragment was cloned into the *Sac*I and *Bgl*II sites of pGL2-Basic (Promega). Linker scanning mutants were generated by PCR as follows. For each construct, two complementary primers carrying the individual mutations were designed (see below). The coding primer was used in combination with the 3′ *Bgl*II primer, whereas the noncoding one was used in combination with the 5′ *Sac*I primer. For both reactions, TPU19 was employed as template and two subfragments were generated, both carrying the mutation at the 5′ and 3′ ends, respectively. The fragments were gel purified, aliquots of both were mixed, combined with the two primers 5′ *Sac*I and 3′ *Bgl*II, and subjected to the second round of PCR. For both rounds of PCR, the following conditions were used: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for 20 cycles. The reactions were carried out as recommended by the manufacturer's specifications (Perkin-Elmer Cetus). The resulting fragments were digested with *Sac*I and *Bgl*II and ligated into the *Sac*I and *Bgl*II sites of pGL2-Basic. The integrity of the individual mutants was confirmed by DNA sequence analysis.

Oligonucleotides used for in vitro mutagenesis. The oligonucleotides used for in vitro mutagenesis are listed in Table 1.

Generation of probes used for binding studies. The probes used in Fig. 3, 4, and 5C were generated by PCR using the wild-type and mutant expression plasmids as template and the 5′ radiolabeled primers TO-818 and TO-701 (for Fig. 3 and 4 probes) and TO-914 and TO-915 (for Fig. 5C probes). Base pair changes introduced in these primers were included to eliminate protein binding to sites (see below) which would have interfered with the analysis. Probes used for DNase I protection studies were also prepared by PCR using the plasmid TPU19 as template and the primers TO-728 and TO-701; only one of the primers was radiolabeled. The DNA sequences of the primers are listed in Table 2.

DNA-protein-binding studies. Nuclear extracts from HUVE cells were prepared as described previously (32). Binding reactions for electrophoretic mobility shift assays were carried out in 10 mM Tris-HCl (pH 7.5)–50 mM NaCl–5 mM dithiothreitol–1 mM EDTA, 0.1 mg of bovine serum albumin per ml–4% glycerol–10 to 50 μg of poly(dI-dC) ml^{-1} using 2×10^4 cpm of the DNA probe and either 5 μg of crude nuclear extracts, 5 ng of p50, 10 ng of p65, or 16 ng of the heterodimer (4 ng of p50 plus 12 ng of p65) in a total volume of 20 μl. The reaction mixtures were incubated for 30 min at room temperature, and protein-DNA complexes were separated on a 4.5% polyacrylamide gel; electrophoresis was carried out in 0.5× Tris-borate-EDTA (TBE) at 8 V cm^{-1}. For experiments in which antibodies were used in the binding reaction, antisera and extract were mixed and incubated for 20 min at room temperature prior to the addition of the DNA probe. Binding reactions for DNase I protection studies were performed as described above, except that the volume of the mixture was raised to 50 μl and the amounts of protein indicated in the figure legends were used. After a 30-min incubation, 50 μl of 10 mM $MgCl_2$–5 mM $CaCl_2$ was added, and then 0.02 U of DNase I (Boehringer) was added. The reaction mixtures were incubated for 1 min at room temperature and were stopped by the addition of 100 μl of 20 mM EDTA–1% SDS–0.25 μg of salmon sperm DNA per ml. The DNA was purified by phenol-chloroform (1:1) extraction and was concentrated by ethanol precipitation. Cleavage products were resolved on an 8% sequencing gel.

Recombinant proteins and antibodies. The recombinant proteins p50 and p65 as well as the antibodies directed against p50, p65, p52, and c-REL were kindly provided by Zhaodan Cao. Antibodies directed against ETS1 and ETS2 were a gift from Cathy Gunther. The anti-GABPα and anti-GABPβ antisera have previously been described (36). Antibodies directed against ATF2 were obtained by injecting rabbits with recombinant ATF2 proteins expressed and purified from *Escherichia coli*.

TABLE 1. Oligonucleotides used for in vitro mutagenesis in this study

Oligonucleotide	Primer[a]	Sequence[b]
5′ *Sac*I primer	TO-475	GCCTGCCTCgagctcCCAAAGTGGTGGGAT
3′ *Bgl*II primer	TO-474	GGGTATCACTGCTagatctGTCTCAGGTCA
LS42	TO-487 (c)	AGGAAGCggcggccgccATAAAAGGGCCTCAGCCG
	TO-488 (nc)	GCCCTTTTATggcggccgccGCTTCCTGTGAATAATAGG
LS43	TO-489 (c)	CCTATTATTggcggccgccAATCCCTCCTATAAAAGG
	TO-490 (nc)	AGGGATTggcggccgccAATAATAGGAGGATATTG
LS44	TO-491 (c)	CAATATCCggcggccgccCACAGGAAGCAATCCCTC
	TO-492 (nc)	TCCTGTGggcggccgccGGATATTGTCCACATCCA
LS45	TO-493 (c)	GGATGTGggcggccgccTCCTATTATTCACAGGAAG
	TO-494 (nc)	AATAATAGGAggcggccgccCACATCCAGTAAAGAGG
LS46	TO-495 (c)	CCTCTTTggcggccgccGACAATATCCTCCTATTATTC
	TO-496 (nc)	TATTGTCggcggccgccAAAGAGGAAATCCCCAAT
LS47	TO-497 (c)	TTGGGGAggcggccgccACTGGATGTGGACAATATC
	TO-498 (nc)	ATCCAGTggcggccgccTCCCCAATGGCATCCAAA
LS48	TO-499 (c)	TTGGATGggcggccgccTTTCCTCTTTACTGGATGTGG
	TO-500 (nc)	AAAGAGGAAAggcggccgccCATCCAAAAACTTTCCCGG
LS49	TO-501 (c)	GGGAAAGggcggccgccCCATTGGGGATTTCCTCTTTAC
	TO-502 (nc)	CCAATGGggcggccgccCTTTCCCGGGAATATCCACG
LS50	TO-503 (c)	GGATATTggcggccgccTTTTTGGATGCCATTGGGG
	TO-504 (nc)	CATCCAAAAAggcggccgccAATATCCACGATGCTTA
LS51	TO-505 (c)	TAAGCATggcggccgccCCCGGGAAAGTTTTTGGATG
	TO-506 (nc)	TCCCGGGggcggccgccATGCTTAAAATTACAATGATG
LS52	TO-507 (c)	ATTGTAAggcggccgccCGTGGATATTCCCGGGAAAG
	TO-508 (nc)	ATATCCACGggcggccgccTTACAATGATGTCAGAAAC
LS53	TO-509 (c)	GTTTCTGACggcggccgccTTTTAAGCATCGTGGATATTCCc
	TO-510 (nc)	CGATGCTTAAAAggcggccgccGTCAGAAACTCTGTCTCTt
LS54	TO-511 (c)	GAGACAGggcggccgccATCATTGTAATTTTAAGCATC
	TO-512 (nc)	TTACAATGATggcggccgccCTGTCTCTTGAAGCTACT
LS55	TO-533 (c)	AGTAGCTTggcggccgccAGTTTCTGACATCATTGTAAT
	TO-534 (nc)	GTCAGAAACTggcggccgccAAGCTACTTCACCTTTGTCC
LS130	TO-652 (c)	GgtGATATTCCCGGGAAAGTTTTTGGATG
	TO-653 (nc)	CATCCAAAAACTTTCCCGGGAATATCacC
LS131	TO-654 (c)	GTGtcTATTCCCGGGAAAGTTTTTGGATG
	TO-655 (nc)	CATCCAAAAACTTTCCCGGGAATAgaCAC
LS132	TO-656 (c)	GTGGAgcTTCCCGGGAAAGTTTTTGGATG
	TO-657 (nc)	CATCCAAAAACTTTCCCGGGAAgcTCCAC
LS133	TO-658 (c)	GTGGATAggCCCGGGAAAGTTTTTGGATG
	TO-659 (nc)	CATCCAAAAACTTTCCCGGGccTATCCAC
LS110	TO-622 (c)	GTGGATATTaaCGGGAAAGTTTTTGGATG
	TO-623 (nc)	CATCCAAAAACTTTCCCGttAATATCCAC
LS111	TO-624 (c)	GTGGATATTCCatGGAAAGTTTTTGGATG
	TO-625 (nc)	ATCCAAAAACTTTCCatGGAATATCCAC
LS112	TO-626 (c)	GTGGATATTCCCGttAAAGTTTTTGGATG
	TO-627 (nc)	CATCCAAAAACTTTaaCGGGAATATCCAC
LS113	TO-628 (c)	GTGGATATTCCCGGGccAGTTTTTGGATG
	TO-629 (nc)	CATCCAAAAACTggCCCGGGAATATCCAC
LS114	TO-630 (c)	GTGGATATTCCCGGGAActTTTTTGGATG
	TO-631 (nc)	CATCCAAAAAagTTCCCGGGAATATCCAC
LS120	TO-634 (c)	GGGAAAGggTTTGGATGCCATTGGGG
	TO-635 (nc)	CCCCAATGGCATCCAAAccCTTTCCC
LS121	TO-636 (c)	GGGAAAGTTggTGGATGCCATTGGGG
	TO-637 (nc)	CCCCAATGGCATCCAccAACTTTCCC
LS122	TO-638 (c)	GGGAAAGTTTTgtGATGCCATTGGGG
	TO-639 (nc)	CCCCAATGGCATCacAAAACTTTCCC
LS123	TO-640 (c)	GGGAAAGTTTTTGtcTGCCATTGGGG
	TO-641 (nc)	CCCCAATGGCAgaCAAAAACTTTCCC
LS124	TO-642 (c)	GGGAAAGTTTTTGGAgtCCATTGGGG
	TO-643 (nc)	CCCCAATGGacTCCAAAAACTTTCCC

[a] c, coding; nc, noncoding.
[b] Letters in lowercase designate introduced nucleotide changes.

RESULTS

Identification of a TNF-α-inducible DNase I-hypersensitive site within the E-selectin promoter. DNase I-hypersensitive sites are often associated with regulatory DNA sequences involved in transcriptional control. Moreover, the appearance of DNase I-hypersensitive sites correlates well with gene activation and binding of transcriptional regulatory proteins (for reviews, see references 12 and 17). In an attempt to identify promoter elements responsible for the cytokine-induced expression of the E-selectin gene, we compared the distributions of DNase I-hypersensitive sites under conditions in which the gene was either active or inactive. Nuclei were

TABLE 2. Sequences of probes and primers used for generating probes for binding studies

Oligonucleotide (description)	Sequence[a]
TO-701 (mutation in NF-ELAM1 site)	CAGAGTTTCTGACAgatcTGTAA
TO-818 (mutation in NF-κB site 3)	CCAGTAAAGAGGAAggatCCAATGG
TO-914 (mutation in NF-κB site 1)	GTAATTTTAAGCATCGTttATATgCC
TO-915 (mutation in NF-κB site 3)	AAAGAGGAAggCatCAATGGCA
TO-728	TTTATAGGAGGGATTggatccTGTGA
Site 1 + 2	
TO-866	gatcTCGTGGATATTCCCGGGAAAGTTTTTG
TO-867	gatcCAAAAACTTTCCCGGGAATATCCACGA
Site 1	
TO-862	gatctCGTGGATATTCCCGGatcCGTGGATATTCCCG
TO-863	gatcCGGGAATATCCACGgatCCGGGAATATCCACGA
Site 2	
TO-864	gatctCGGGAAAGTTTTTGGatCCGGGAAAGTTTTTg
TO-865	gatcCAAAAACTTTCCCGGatCCAAAAACTTTCCCGa
SITE 1	
TO-952	AAGCATCGTGGATATTCCCGGcAcAGcT
TO-953	AgCTgTgCCGGGAATATCCACGATGCTT
SITE 2	
TO-950	tATATgCCCGGGAAAGTTTTTGtATtCC
TO-951	GGaATaCAAAAACTTTCCCGGGcATATa
SITE 3	
TO-958	GATGCCATTGGGGATTTCCTCTTTACTG
TO-959	CAGTAAAGAGGAAATCCCCAATGGCATC

[a] Lowercase letters indicate introduced nucleotide changes.

isolated either from uninduced HUVE cells or from cells that had been treated with TNF-α for 4 h. DNase I-hypersensitive sites were identified within an 8-kb *Eco*RI fragment bearing 2.7 kb downstream of the E-selectin transcriptional start site and 5.3 kb of upstream sequences (9) (Fig. 1A). A TNF-α-induced DNase I-hypersensitive site was detected approximately 100 bp upstream of the transcriptional start site (Fig. 1B). Additional DNase I-hypersensitive sites were detected further upstream; however, none of them were dependent on TNF-α treatment (data not shown). These data pinpoint the proximal promoter region of the E-selectin gene as potentially important for cytokine-induced gene activation.

Two promoter regions are required for TNF-α-induced expression of the E-selectin gene. To explore whether a DNA fragment encompassing the DNase I-hypersensitive site is capable of conferring TNF-α-induced expression to a heterologous gene, a 782-bp fragment from the E-selectin gene (−730 to +52) was fused to the coding sequences of the firefly luciferase gene. This reporter plasmid was transfected into HUVE cells, and luciferase activity in untreated cells as well as in cells that had been exposed to TNF-α for 4 h was measured. This 782-bp fragment was sufficient to confer TNF-α responsiveness to the luciferase gene (wt in Fig. 2C). To more precisely delineate the region required for TNF-α inducibility, we created several deletion constructs and tested them by transfection in HUVE cells. These studies revealed that a plasmid containing the proximal 170 bp of the E-selectin promoter was induced to the same extent by TNF-α as was the original −730 construct. In contrast, a DNA fragment retaining only 85 bp of 5′ flanking DNA (−85 to +52) was unresponsive to TNF-α treatment (data not shown), indicating that a region essential for TNF-α inducibility resides between residues −170 and −85.

To identify *cis*-regulatory sequences required for cytokine-induced expression, we performed a detailed mutational analysis of the region between −169 and −30 (Fig. 2A and B). In vitro mutagenesis was used to prepare a systematic series of promoter mutants, each differing by only 10 bp from the wild-type construct (−730 to +52). The plasmids were transfected into HUVE cells, and luciferase activity in the presence and absence of TNF-α was determined. As shown in Fig. 2C, most of these promoter mutants responded to TNF-α treatment in a manner similar to that of the wild-type construct. The integrity of two regions of the E-selectin promoter, −129 to −110 (LS51 and LS50) and −99 to −80 (LS48 and LS47), was essential for TNF-α-induced expression. The region from −99 to −80 corresponds to an NF-κB site that is also important for inducibility by IL-1 (38). The region from −129 to −110 represents a previously unidentified regulatory element in the E-selectin promoter. In addition, the sequence between −159 and −140, which corresponds to a previously identified NF-ELAM1/ATF2 site (19), contributed to maximal induction by TNF-α. Finally, three promoter mutants, LS44, LS49, and LS52, gave rise to elevated basal expression, with LS44 also showing increased expression in the presence of TNF-α. In subsequent studies, we focused on the newly defined regulatory region that appeared to be most crucial for TNF-α-induced expression (−129 to −110).

Identification of a new NF-κB-binding site within the E-selectin promoter. To identify nuclear proteins that specifically interact with the E-selectin promoter following TNF-α activation, we performed electrophoretic mobility shift assays using extracts prepared from TNF-α-treated or untreated HUVE cells (Fig. 3). A promoter fragment extending from −162 to −75 and carrying mutations in the NF-ELAM1 site (−153 to −146) and the NF-κB site (−94 to −85; see Materials and Methods) was bound by two nuclear activities. The more slowly migrating protein-DNA complex (complex I) was formed only with extracts from TNF-α-treated cells (lanes 3 and 4, 3 and 8 h of TNF-α treatment, respectively), suggesting that the region critical for expression in the presence of TNF-α may be bound by an inducible activity. In contrast, the more rapidly migrating protein-DNA complex (complex II; Fig. 3A) was observed with both untreated (lane 2) and TNF-α-treated (lanes 3 to 4) cell extracts. Apparently, this complex corresponds to a constitutively active nuclear factor.

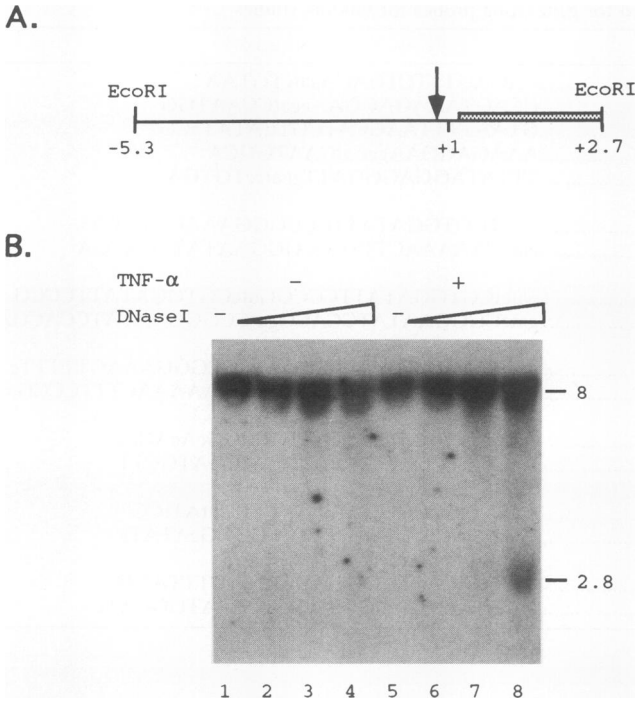


FIG. 1. Detection of a TNF-α-inducible DNase I-hypersensitive site in the E-selectin promoter. (A) Restriction map of the E-selectin promoter. An 8-kb *Eco*RI fragment (−5.3 to +2.7) was chosen to identify DNase I-hypersensitive sites in the proximity of the transcriptional start site (+1). The *Stu*I-*Eco*RI fragment (dotted bar) was used as probe for indirect labeling. The arrow marks the position of the DNase I-hypersensitive site induced by treatment of cells with TNF-α. (B) Southern blot. Nuclei prepared from untreated HUVE cells (lanes 1 to 4) or from cells that had been incubated with TNF-α for 4 h (lanes 5 to 8) were either untreated (lanes 1 and 5) or treated with increasing amounts of DNase I (indicated by the wedges; lanes 2 and 6, 0.75 U ml^{-1}; lanes 3 and 7, 1.5 U ml^{-1}; lanes 4 and 8, 3 U ml^{-1}). After *Eco*RI digestion, the fragments were separated on a 0.8% agarose gel, transferred onto nitrocellulose, and probed with a *Stu*I-*Eco*RI fragment (dotted bar in panel A). Numbers on the right are given in kilobases.

Stimulated expression in response to cytokines often requires the activity of the inducible transcription factor NF-κB (1, 4). In fact, the DNA sequences in the region from −129 to −110 of the E-selectin promoter shared some similarity with previously identified NF-κB binding sites (23). However, the presence of two inverted GGAA motifs within region −129 to −110 suggested that the element critical for TNF-α-induced expression might instead be recognized by an ETS protein (for a review, see reference 21). Hence, in an initial attempt to characterize the protein(s) involved in complex I, we used antibodies directed against individual members of the NF-κB or ETS families of transcription factors. The formation of complex I was completely abolished when the nuclear extracts were preincubated with antibodies against the NF-κB family member p50 or p65 (Fig. 3B, lanes 5 and 6), whereas no effect on complex I formation was observed when the extract was incubated with antisera directed against p52 (lane 3), c-REL (lane 4), ETS1 (lane 7), ETS2 (lane 8), GABPα (lane 9), GABPβ (lane 10), or ATF2 (lane 11) or with preimmune serum (lane 12). The migration of complex II was not affected by any of the antisera (lanes 3 to 12). These data indicated that complex I is mediated by an inducible form of NF-κB consisting of p50 and p65 subunits.

Region −129 to −110 carries two closely spaced NF-κB binding sites. To test directly whether NF-κB (p50/p65) can bind to positions −129 to −110 of the E-selectin promoter region and to investigate whether this binding is functionally relevant to promoter activity, we performed electrophoretic mobility shift assays using recombinant proteins and DNA probes bearing either the wild-type sequence or the 10-bp mutations LS51, -50, and -49, respectively (Fig. 4A). The probes extended from position −162 to −75; however, mutations were introduced into the NF-ELAM1 site (−153 to −146) and the previously identified NF-κB site (−94 to −85; see Materials and Methods). Homodimers of either p50 or p65 (Fig. 4B, lanes 2 and 6), as well as the p50/p65 heterodimer (lane 10), bound to the wild-type DNA probe, confirming the presence of another NF-κB binding site within this promoter fragment. The DNA probe bearing the LS50 mutation completely abolished binding of both the p50 and p65 homodimers (lanes 4 and 8) as well as that of the p50/p65 heterodimer (lane 12). These observations correlated well with the lack of cytokine inducibility of the LS50 mutant (Fig. 2). As expected, the probe bearing the LS49 mutation served as a good substrate for NF-κB binding (Fig. 4B, lanes 5, 9, and 13). The LS51 probe also bound recombinant NF-κB to an extent similar to that of the wild-type probe (lanes 3, 7, and 11), despite the 60% reduction in TNF-α-induced expression seen with LS51 (Fig. 2).

Two possible explanations might account for the latter result. First, perhaps only bp −119 to −110 (targeted by LS50) are crucial for NF-κB binding. This possibility seemed unlikely, since the DNA sequence encompassed by LS50 does not resemble a canonical NF-κB-binding site (23). An alternative explanation would be that region −129 to −110 harbors two NF-κB sites and that the LS50 mutation alters both of them, whereas the LS51 change affects just one site. To test the first possibility, we used an oligonucleotide probe carrying the region between −124 and −111, flanked by arbitrarily chosen DNA sequences. Recombinant p50/p65 failed to bind this DNA probe, indicating that the 10 bp mutated by LS50 does not form an NF-κB-binding site (data not shown).

A close inspection of the DNA sequences in the region from positions −129 to −110 of the E-selectin promoter revealed the presence of two potential NF-κB binding sites, one starting at position −126 and the other starting at position −116 (site 1 and site 2; Fig. 4A). The LS50 mutation should abolish both of these hypothetical sites, whereas the LS51 mutation should affect only the more upstream site. To examine this idea of two adjacent NF-κB sites, we prepared DNA probes containing either site 1 or site 2 and tested them for protein binding in mobility shift assays. Figure 4C illustrates that DNA fragments bearing either both sites (lanes 1 to 4), site 1 (lanes 5 to 8), or site 2 (lanes 9 to 12) served as good substrates for NF-κB binding. All three probes bound all three combinations of NF-κB (p50 homodimer, lanes 2, 6, and 10; p65 homodimer, lanes 3, 7, and 11; or the p50/p65 heterodimer, lanes 4, 8, and 12). Similarly, both sites were also independently bound by the TNF-α-inducible NF-κB activity in nuclear extracts from HUVE cells (data not shown). Taken together, these results indicate that the newly identified regulatory element of the human E-selectin promoter is composed of two NF-κB sites and that the mutations introduced by LS50 impinge on both sites.

The murine E-selectin sequence (2), homologous to region −128 to −103 of the human gene, differs by only 3 bp (GTGGATATTCCCaGaAAAcTTT [lowercase letters indi-

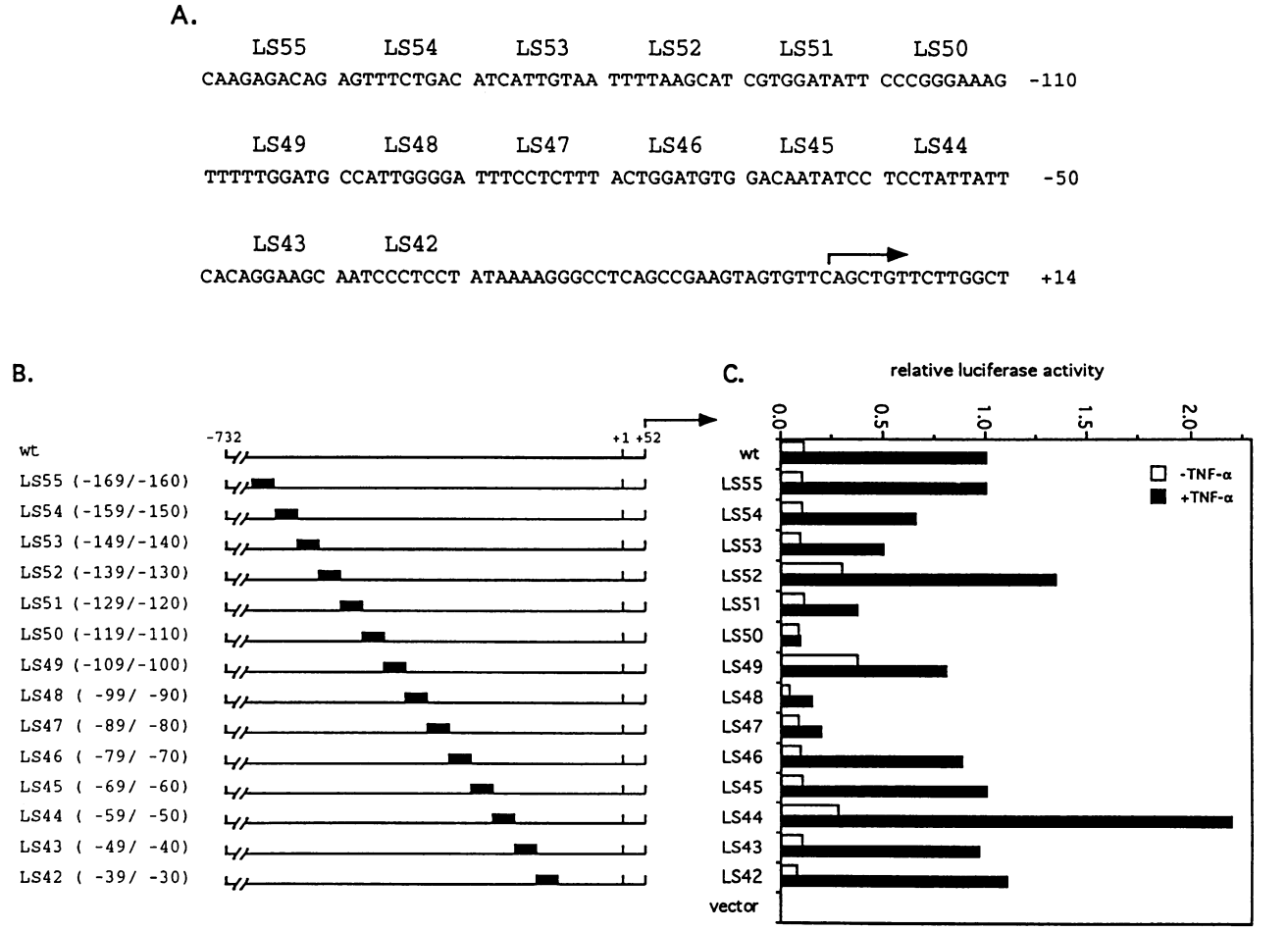


FIG. 2. Identification of promoter elements critical for TNF-α inducibility. (A) Nucleotide sequence of the E-selectin promoter. The nucleotide sequence of the E-selectin promoter extending from −169 to +14 is shown. LS55 to LS42 designate the 10-bp regions that have been substituted by the sequence GGCGGCCGCC in the individual mutants in panel B. (B) Wild-type (wt) and mutant reporter constructs used for transfection studies. The wild-type sequence (−730 to +52) was fused to the luciferase reporter gene. The mutant derivatives (LS55 to LS42) differ by only the 10 bp indicated in panel A from the wild-type construct. (C) Relative luciferase activities obtained with the wild-type and mutant reporter constructs. The individual constructs shown in panel B were transfected into HUVE cells. Luciferase activity was determined 48 h after transfection from either untreated cells (white bars) or from cells that had been exposed to TNF-α for 4 h prior to harvesting (black bars). Numbers on the left axis indicate the relative luciferase activities, the value obtained with the wild-type construct in TNF-α-treated HUVE cells being taken as 1. Each construct was assayed in duplicate in six independent experiments. The values from one representative experiment are shown. Similar relative activities were observed in all other transfections.

cate the nucleotides that differ from the human sequence]). Using mobility shift assays, we observed that the murine element was specifically recognized by both recombinant NF-κB and NF-κB present in nuclear extracts derived from TNF-α-induced HUVE cells (data not shown). These data indicate that cytokine-induced expression of the E-selectin gene in mice is also mediated by NF-κB and probably requires sequences corresponding to the region from positions −128 to −110 of the human promoter.

Site 2 is functionally more important than site 1 and is preferentially occupied by NF-κB. The presence of two closely spaced NF-κB sites (site 1 and site 2) within the E-selectin promoter raised the question of whether both sites are equally important in vivo or whether one of them is preferentially occupied and, hence, more crucial for TNF-α-induced expression. To discriminate between the two possibilities, we performed a more detailed mutational analysis within region −127 to −106 encompassing both NF-κB sites (Fig. 5A). Reporter constructs were created that differed by only 2 bp from the wild-type promoter (−730 to +52) and were assayed for luciferase activity in both TNF-α-induced and uninduced HUVE cells. Two of the mutations within site 2 (LS112 and LS113) significantly reduced promoter activity in response to TNF-α, while another 2-bp mutation within site 2 (LS120) caused a modest reduction in TNF-α-induced expression (Fig. 5B). In contrast, all of the 2-bp mutations within site 1 led to only a modest reduction in TNF-α-induced expression. These results suggest that site 2 is functionally more important than site 1; however, maximal promoter activity required both sites 1 and 2.

We then performed mobility shift assays using wild-type and mutant DNA fragments corresponding to site 2 to compare the transfection data with those of in vitro binding by NF-κB. The probes extented from position −143 to −80 but carried mutations that eliminated binding of NF-κB to sites 1 and 3 (see Materials and Methods). The results of Fig. 5C show that

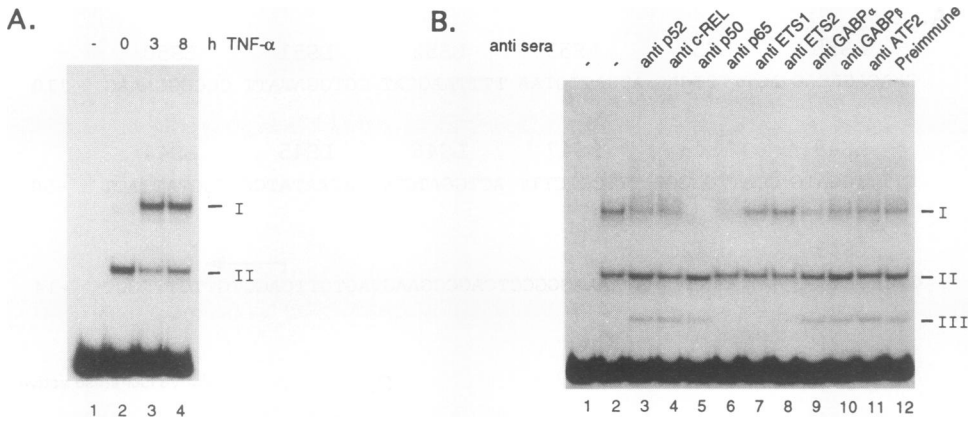


FIG. 3. Characterization of proteins binding to the region from positions −129 to −100 of the E-selectin promoter. (A) Electrophoretic mobility shift assay using crude nuclear extracts. The radiolabeled wild-type promoter fragment containing region −129 to −100 was incubated with nuclear extracts derived from either untreated HUVE cells (lane 2) or from cells that had been exposed to TNF-α for 3 h (lane 3) or 8 h (lane 4). Lane 1 represents the DNA probe without any protein added. Two protein-DNA complexes were observed (complexes I and II). (B) Immunological characterization of the inducible DNA-binding activity. Nuclear extracts from HUVE cells treated with TNF-α for 3 h were incubated with antisera directed against different transcription factors (lanes 3 to 12) prior to the addition of the DNA probe. Lanes: 1, DNA probe without any added extracts; 2, DNA probe incubated with nuclear extracts without any antibodies. Complex III represents an irrelevant DNA binding activity present in some antisera.

the heterodimeric protein p50/p65 bound avidly to the wild-type site 2 sequence (lane 1). The heterodimeric protein p50/p65 failed to bind to the mutant probes (LS112, LS113, and LS120) that had shown reduced TNF-α inducibility in transfected HUVE cells. Moreover, mutants that had no effect on TNF-α inducibility (LS111, LS114, and LS121) bound p50/p65 to the same extent as did the wild-type probe. Probes bearing mutations outside the proposed NF-κB site 2 (LS122 and LS123) also proved to be avid substrates for NF-κB binding. Very similar results were obtained with the homodimeric p50 and p65 proteins (Fig. 5C), although the p50 homodimer seemed to exhibit a greater requirement for the dinucleotide CG (LS111) than did the p65 homodimer or the p50/p65 heterodimer. The concordance of in vitro binding and transactivation in vivo is consistent with the interpretation that TNF-α induction of E-selectin gene expression in endothelial cells entails binding of NF-κB to a region (site 2) located −116 bp upstream from the transcription start site.

All binding studies outlined thus far were carried out using DNA probes that lacked the previously identified NF-κB site (located between residues −94 and −85 and termed site 3; Fig. 6). As another approach to test whether all three potential NF-κB sites could be occupied simultaneously, we performed DNase I footprinting studies using a promoter fragment that carried all three sites. Figure 6 shows that, when exposed to increasing amounts of the p50 homodimer or p50/p65 heterodimer, site 2 and site 3 are occupied whereas site 1 is not. However, in addition to binding to site 2 and site 3, the p65 homodimer was capable of co-occupying all three NF-κB binding sites, although binding of p65 homodimers to site 1 occurred only when very large amounts of protein were used. Furthermore, binding of p65 to this site was completely eliminated when a DNA fragment carrying the LS51 mutation was employed (Fig. 6C, lanes 11 to 14). Finally, no differences in the binding patterns of the p50 homodimer or the p50/65 heterodimer were observed when we compared the wild-type fragment with the LS51 mutant.

To explore the possibility that site 2 was preferentially bound over site 1 as a result of favorable interactions between site 2 and site 3, we monitored binding of NF-κB to a probe (LS48) bearing mutations in site 3. Site 2 is bound equally well by the NF-κB proteins in the absence of an intact site 3, indicating that binding of NF-κB to site 2 is not assisted by NF-κB bound to site 3 (Fig. 6D). This experiment also showed that the p50/p65 heterodimer is capable of interacting with site 1 and site 2 simultaneously at high protein concentrations (lane 13), as was the case for p65 homodimers (lanes 8 and 9). Finally, we examined NF-κB binding to a promoter fragment bearing a mutation in site 2 (LS112). Despite significantly affecting binding of all three NF-κB samples (p65, p50, and p50/65) to site 2, this mutation did not significantly affect binding to either site 1 or site 3 (Fig. 6E). In summary, these data argue that within the context of a larger promoter fragment, sites 2 and 3 are bound with high affinity by either the homodimeric (p50 and p65) or the heterodimeric (p50/p65) forms of NF-κB. In contrast, site 1 appears to be recognized with lower affinity and is preferentially bound by the p65 homodimer. These observations are consistent with the effects on TNF-α-mediated gene activation in that mutations within sites 2 and 3 are more detrimental than mutations in site 1 (Fig. 2 and 5). These data also agree with the finding that two protein-DNA complexes are observed in mobility shift assays employing DNA probes carrying both sites 1 and 2 with large amounts of p65 (data not shown). Finally, footprinting studies using recombinant forms of NF-κB (p50, p65, and p50/p65) failed to detect any cooperative interactions between NF-κB proteins bound to the three sites within the E-selectin proximal promoter.

To evaluate the relative binding affinities of the NF-κB proteins to the three sites independently, we did competition studies with individual sites. The protein-DNA complexes formed with site 3 (Fig. 7, lane 1) by either the p50 or the p65 homodimer or the p50/p65 heterodimer were challenged with oligonucleotides carrying site 1 (lanes 2 to 5), site 2 (lanes 6 to 9), or site 3 sequences (lanes 10 to 13). As seen with the DNAse I footprinting experiments, sites 2 and 3 showed the highest binding affinities for all three NF-κB proteins and bound all three proteins similarly (Fig. 7A). In contrast, 10 times more site 1 sequence was needed to inhibit p50 binding than either site 2 or site 3 sequence. Site 1 was also less effective than the other sites in inhibiting p50/p65 binding.

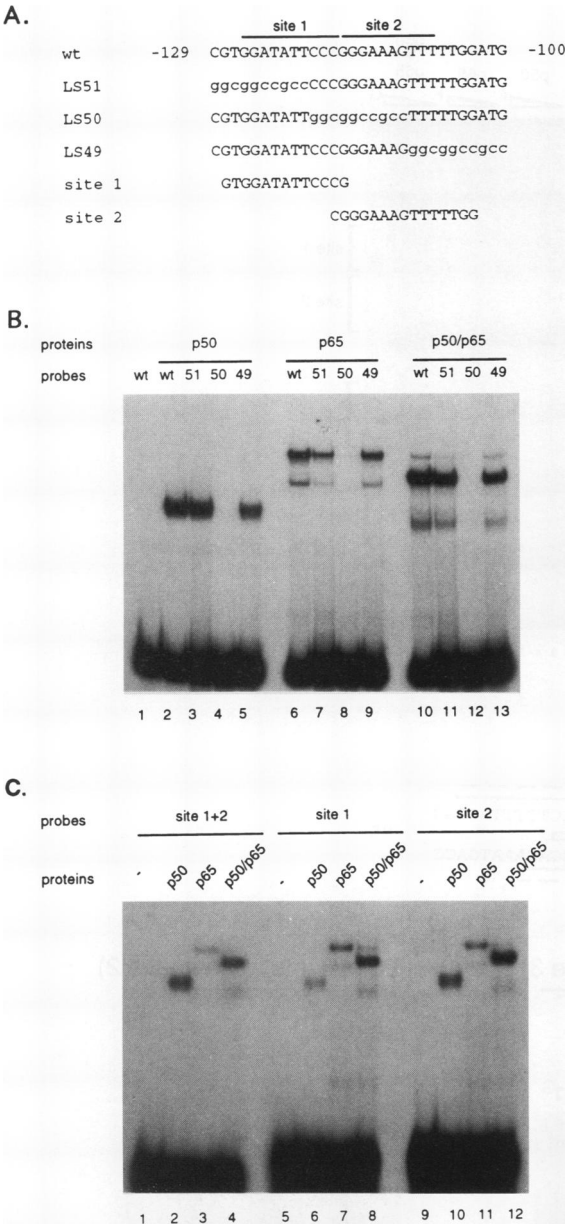


FIG. 4. Analysis of NF-κB binding to the region from positions −129 to −100 of the E-selectin promoter. (A) DNA sequences of the probes used for protein binding studies. The wild-type DNA probe (wt) carries the promoter region critical for TNF-α inducibility. LS51, LS50, and LS49 differ by 10 bp from the wild-type sequence; lowercase letters represent the changed nucleotides. The probes were generated by PCR as described in Materials and Methods. Two potential NF-κB binding sites (site 1 and site 2) identified by sequence analysis are indicated. (B) Electrophoretic mobility shift assay with wild-type and mutant DNA probes. Recombinant proteins p50 homodimer (lanes 2 to 5), p65 homodimer (lanes 6 to 9), and the heterodimer p50/p65 (lanes 10 to 13) were incubated with the individual DNA probes designated above each lane. The relevant differences in sequences between the four probes are given in panel A. No proteins were added to the binding reaction mixture in lane 1. (C) Electrophoretic mobility shift assay using site 1- and site 2-containing oligonucleotides. The recombinant proteins indicated above each lane were incubated with a radiolabeled oligonucleotide probe carrying either both NF-κB sites (lanes 2 to 4), a probe with only site 1 (lanes 6 to 8), or a probe with only site 2 (lanes 10 to 12). No proteins were added for lanes 1, 5, and 9.

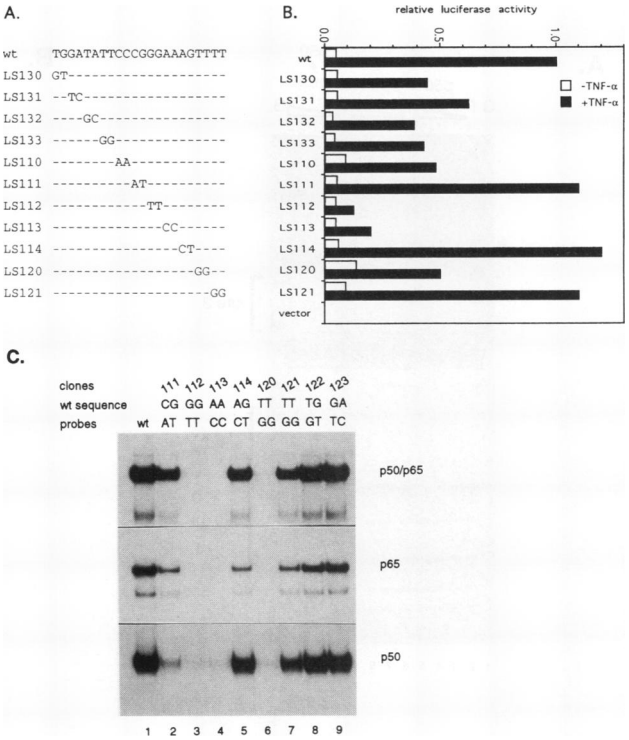


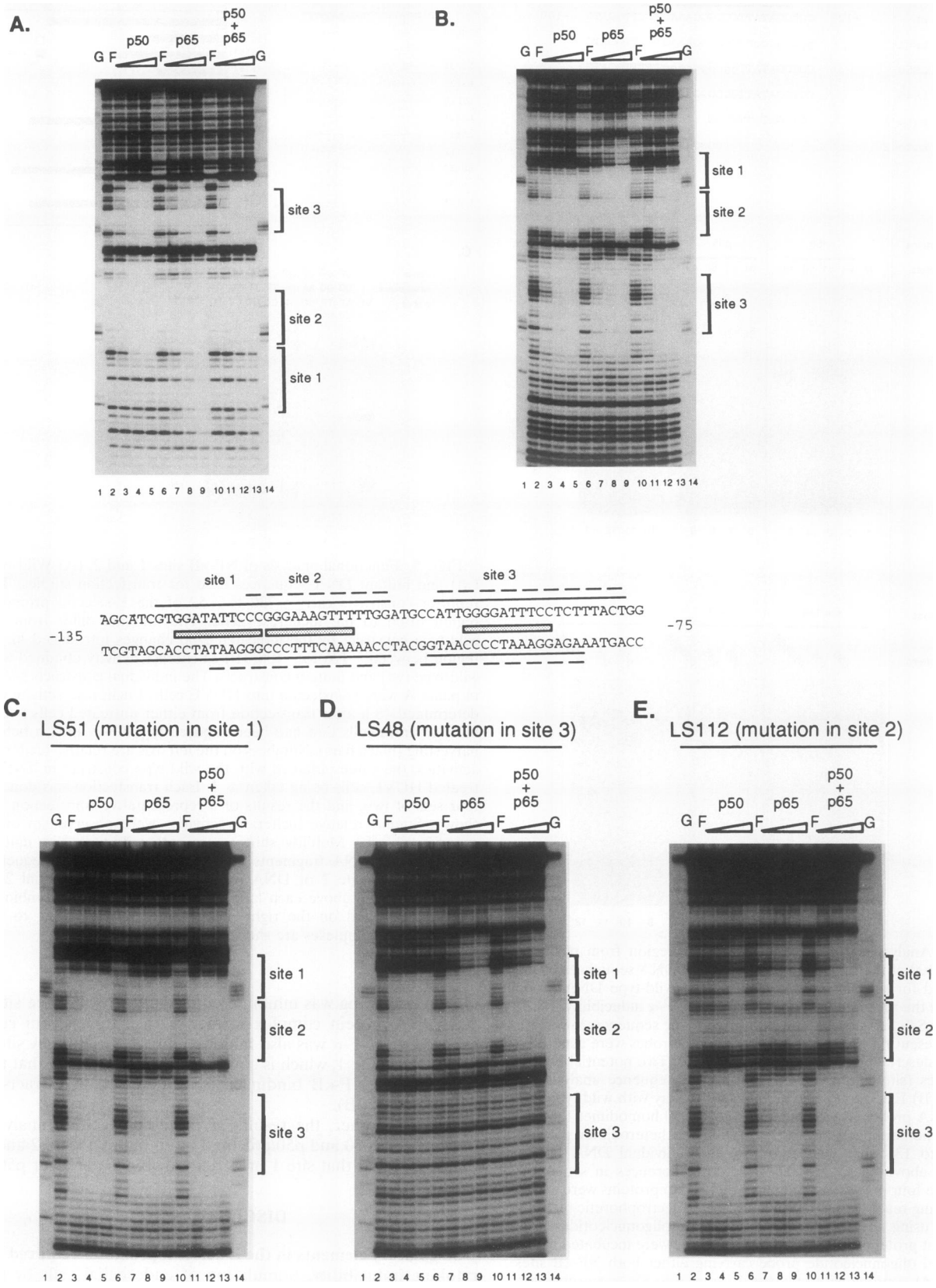
FIG. 5. Functional analysis of NF-κB sites 1 and 2. (A) Wild-type (wt) and mutant DNA sequences used for transfection studies. The reporter constructs carry −730 to +52 of the E-selectin promoter region fused to the luciferase gene. The mutants differ from the wild-type construct by the indicated 2-bp changes introduced in the region between −126 to −106. (B) Luciferase activity obtained with wild-type (wt) and mutant constructs. The individual constructs shown in panel A were transfected into HUVE cells. Luciferase activity was determined 48 h after transfection from either untreated cells (white bars) or from cells that had been exposed to TNF-α for 4 h before harvesting (black bars). Numbers on the left axis are relative luciferase activities, the value obtained with the wild-type construct in TNF-α-treated HUVE cells being taken as 1. Each transfection was done in four sets of two, and the results of a representative transfection are shown. Similar relative luciferase activities were observed in other experiments. (C) Mobility shift assays with wild-type and mutant NF-κB site 2. DNA fragments representing the wild-type sequence (wt) of NF-κB site 2 or DNA probes carrying the individual 2-bp mutations shown above each lane were incubated with recombinant proteins indicated on the right. Only bands corresponding to the protein-DNA complexes are shown.

Only p65 binding was inhibited equivalently by all three sites. The DNA-protein complex formed with extracts from cells exposed to TNF-α was also inhibited more efficiently by site 2 or 3 than by site 1, which is consistent with the finding that the predominant NF-κB binding activity detected in extracts is p50/p65 (Fig. 3B).

Taken together, the results of these competition analyses indicate that p50 and p50/p65 bind more tightly to site 2 and 3 than site 1 and that site 1 preferentially binds p65 over p50.

DISCUSSION

Regulatory elements in the E-selectin promoter required for cytokine inducibility. Stimulation of endothelial cells by the inflammatory cytokines such as TNF-α and IL-1 results in the rapid transcriptional induction of the gene encoding E-selectin


A.
p50
p65
p50 + p65
G F F F G
site 3
site 2
site 1
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B.
p50
p65
p50 + p65
G F F F G
site 1
site 2
site 3
1 2 3 4 5 6 7 8 9 10 11 12 13 14
site 1
site 2
site 3
AGCATCGTGGATATTCCCGGGAAAGTTTTTGGATGCCATTGGGGATTTCCTCTTTACTGG
-135
-75
TCGTAGCACCTATAAGGGCCCTTTCAAAAACCTACGGTAACCCCTAAAGGAGAAATGACC
C.
LS51 (mutation in site 1)
p50
p65
p50 + p65
G F F F G
site 1
site 2
site 3
1 2 3 4 5 6 7 8 9 10 11 12 13 14
D.
LS48 (mutation in site 3)
p50
p65
p50 + p65
G F F F G
site 1
site 2
site 3
1 2 3 4 5 6 7 8 9 10 11 12 13 14
E.
LS112 (mutation in site 2)
p50
p65
p50 + p65
G F F F G
site 1
site 2
site 3
1 2 3 4 5 6 7 8 9 10 11 12 13 14

(5). E-selectin increases the adhesion of neutrophils to the endothelium, which constitutes an important early step in the sequence of events leading to the extravasation of neutrophils through the endothelium to sites of inflammation (for reviews, see references 7 and 30). E-selectin expression is regulated primarily at the level of transcription. Here, we describe the identification of a new regulatory DNA element in the human E-selectin promoter that is essential for TNF-α-induced gene expression in HUVE cells. The element is composed of two closely spaced NF-κB binding sites, designated site 1 (−126 to −117) and site 2 (−116 to −108). A third NF-κB site located further downstream, site 3 (−94 to −85), had previously been shown to be essential for IL-1-induced activation of the E-selectin promoter (19, 38). Our data reveal that sites 2 and 3 contribute significantly to the TNF-α-induced expression of the E-selectin gene; however, all three sites are required for maximal promoter activity. A complete loss of induction was observed when sites 1 and 2 were mutated, demonstrating that activation of the E-selectin gene requires more than one NF-κB-binding site.

Our data did not reveal any other regulatory elements that are absolutely essential for TNF-α-induced expression. These results differ from earlier studies that revealed a substantive requirement for two additional *cis*-regulatory DNA sequences that bind the NF-ELAM1 and NF-ELAM2 nuclear factors (19). The bases for the discrepancies between the two results are unknown but may reflect differences in (i) the experimental protocol, (ii) the two cell-types (HUVE cells versus the transformed endothelial cell line IE-7), or (iii) the two cytokines (TNF-α versus IL-1) used.

NF-κB sites 2 and 3 are preferentially occupied relative to site 1. The observation that NF-κB site 2 mutations compromise promoter activity more profoundly than do mutations within site 1 is surprising, since site 1 better resembles a canonical NF-κB binding site than does site 2 (Fig. 7B) (23). Indeed, site 2 is exceptional in that the two C residues typically found at the 3′ end of the NF-κB recognition sequence are replaced by two T residues. However, consistent with functional data, DNase I protection studies clearly showed that, relative to site 1, sites 2 and 3 are preferentially bound by NF-κB. The competition studies also clearly demonstrate that the binding affinities of p50, p50/p65, and the NF-κB binding activity induced in HUVEC extracts by TNF-α for sites 2 and 3 are greater than those for site 1. Hence, the results suggest that sites 1 and 2 may be competing sites that at limiting protein concentrations are probably not occupied simultaneously, either because of sterical hinderance and/or because site 2 represents a higher-affinity binding site than 1. An alternative explanation would be that NF-κB binding to site 3 facilitates binding to site 2. However, DNase I protection studies on DNA fragments bearing mutations in either one of the two sites revealed that sites 2 and 3 bind NF-κB with similar affinities and that binding to one site does not influence binding to the other. Finally, mutation of site 2 neither impeded nor greatly potentiated binding to site 1. In contrast to p50 and p50/p65 binding, the p65 homodimers bind equivalently to all three sites, as revealed by competition studies and DNase I footprint analysis. Hence, for reasons that we do not fully understand, the promoter context seems to facilitate NF-κB binding to site 2, whereas site 1 can be occupied only by a p65 homodimer or a p50/p65 heterodimer at high protein concentrations.

Another interesting outcome of our studies is the observation that mutations within site 2 (LS112 and LS113) or site 3 (LS47 and LS48) have a more detrimental effect on TNF-α-induced expression than one would predict if the two sites simply represent redundant elements. These results suggest that the NF-κB proteins bound to these two sites activate the E-selectin promoter in concert, despite the lack of cooperativity seen in in vitro DNA-binding studies.

Although the reason why occupancy of multiple NF-κB sites is essential for expression of the E-selectin gene is unknown, it is tempting to speculate that this requirement reflects the formation of macromolecular complex of transcription factors at the promoter. It is possible that NF-κB molecules bound to these sites interact with one another and with factors bound to other sites, such as the NF-ELAM-1/ATF-2 sites (22) and that the transcriptional activation domains of these factors in combination present an activation surface to attract RNA polymerase II and the general transcription factors to the promoter (37). A more detailed analysis in which the positions of the various transcription factor binding sites are changed relative to one another is essential to determine if indeed a multiprotein transcription factor complex is assembled at the E-selectin promoter, as has been described for some other eukaryotic promoters (11).

The number and arrangement of NF-κB binding sites may determine the onset of E-selectin, VCAM-1, and ICAM-1 expression. Inflammatory cytokines such as TNF-α and IL-1 stimulate the transcription of the genes encoding two other cell adhesion molecules, ICAM-1 and VCAM-1 (31, 34). The kinetics of transcriptional induction for the three adhesion molecules after cytokine treatment differ substantially (for reviews, see references 8 and 26). The E-selectin gene is activated very rapidly, with maximal concentrations of cell surface protein accumulating 3 to 4 h postinduction. Basal levels of E-selectin return 16 to 24 h later even in the continuous presence of the inductive cytokine. VCAM-1 appears on the cell surface 4 to 6 h following cytokine presentation, reaches maximal expression at 12 to 18 h, and then gradually declines over a period of days. Maximal levels of ICAM-1 appear on the cell surface 18 to 24 h postinduction

FIG. 6. DNase I protection analysis of NF-κB binding. (A) DNase I protection analysis. The wild-type promoter fragment (−162 to −25) radiolabeled on one strand was incubated with increasing amounts of recombinant protein (lanes 3 to 5, 15, 45, and 150 ng of p50, respectively; lanes 7 to 9, 30, 90, and 300 ng of p65, respectively; lanes 11 to 13, 15/45, 40/120, and 120/360 ng of p50/p65, respectively). Lanes 2, 6, and 10 contain the DNase I digestion products of fragments that had not been incubated with protein (F). Lanes 1 and 14 represent Maxam-Gilbert sequencing reactions (25) of the same DNA fragment (G). The locations of NF-κB sites 1, 2, and 3 are indicated (site 3 corresponds to the previously identified NF-κB site). (B) DNase I protection analysis as described for panel A, except that the complementary strand was radiolabeled. The DNA sequence of the promoter region is given below. Stippled lines designate the regions that are protected by the homodimeric protein p50 or the heterodimer p50/p65. Solid lines indicate the areas that are protected by the p65 homodimer. The DNA sequences most closely matching the canonical NF-κB binding sites are marked by the bars between the two DNA strands. (C) DNase I protection analysis using a DNA fragment which carried the LS51 mutation in NF-κB site 1. The lower strand of the DNA fragment (−162 to −25) was radiolabeled and incubated with increasing amounts of recombinant protein (lanes 3 to 5, 30, 90, and 270 ng of p50, respectively; lanes 7 to 9, 60, 180, and 540 ng of p65, respectively; lanes 11 to 13, 20/60, 60/180, and 180/540 ng of p50/p65, respectively). Other designations correspond to the ones shown in panel A. (D and E) DNase I protection analyses as described for panel C, except that DNA fragments carrying mutations in site 3 (LS48 mutant) and site 2 (LS112 mutant) were employed.

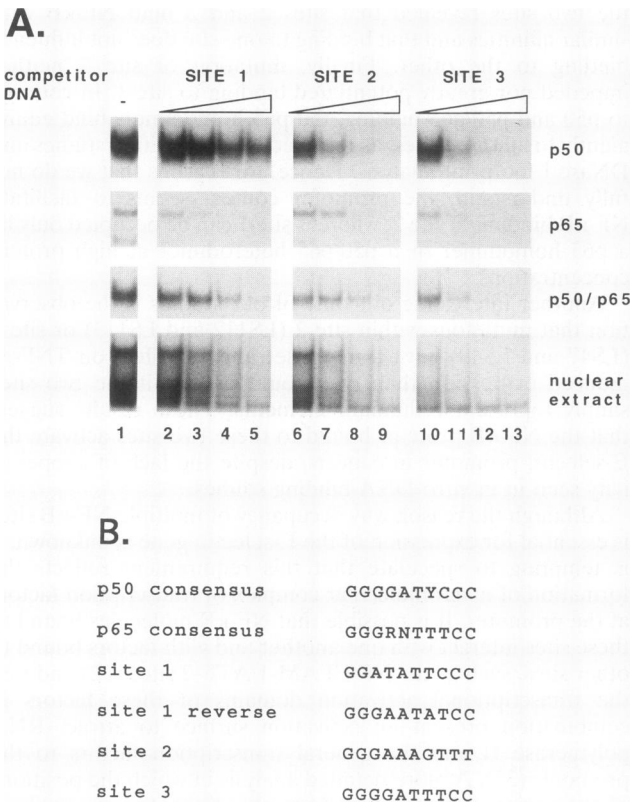


FIG. 7. Determination of the relative binding affinities of NF-κB proteins for sites 1, 2, and 3. (A) Competitive DNA binding studies. Recombinant proteins (the p50 and p65 homodimers and the p50/p65 heterodimer) or crude nuclear extract derived from TNF-α-induced HUVE cells was incubated with a radiolabeled oligonucleotide encompassing the site 3 NF-κB sequence. Only the protein-DNA complexes are shown in all lanes. Lane 1 is a protein-DNA complex formed in the absence of any competitor. For all other lanes, various amounts of unlabeled oligonucleotides were included in the binding reaction mixtures (lanes 2 to 5, SITE 1; lanes 6 to 9, SITE 2; lanes 10 to 13, SITE 3; the DNA sequences are given in Table 2). For the recombinant proteins, 0.3 pmol (lanes 2, 6, and 10), 1 pmol (lanes 3, 7, and 11), 3 pmol (lanes 4, 8, and 12), and 9 pmol (lanes 5, 9, and 13) of competitor oligonucleotide were used. The autoradiogram for p50 binding was exposed for a longer period of time to emphasize the differences in binding affinity for the three sites. For the crude nuclear extract, 0.1 pmol (lanes 2, 6, and 10), 0.3 pmol (lanes 3, 7, and 11), 1 pmol (lanes 4, 8, and 12), and 3 pmol (lanes 5, 9, and 13) of competitor oligonucleotides were included. In the case of the crude nuclear extract, three protein-DNA complexes were observed; the fastest migrating complex represents a nonspecific DNA binding activity present in extracts. The two more slowly migrating bands are specifically inhibited by oligonucleotides having the NF-κB recognition sequence; the faster migrating one of the two is most likely a proteolytic degradation product of the endogenous NF-κB binding activity and was not detected in all experiments. (B) Alignment of NF-κB binding sites. The sequences of the three NF-κB sites identified in the E-selectin promoter are aligned with the consensus sequences of the p50 and p65 binding sites which had been described previously (23). Both strands of site 1 resemble an NF-κB recognition motif.

and remain at the same high level as long as cytokines are present (34). The cytokine-induced expression of all three promoters requires the activity of NF-κB. However, the three genes differ in the numbers and organization of NF-κB binding sites. Cytokine-induced expression of E-selectin requires the integrity of all three NF-κB sites (the present work), the VCAM-1 promoter contains two NF-κB binding sites, both of which are required for maximal induction (20, 28, 29, 35), and the ICAM-1 promoter is characterized by one essential NF-κB and one essential CEB/P binding site (19a). A simple interpretation of these observations is that E-selectin transcription is triggered more rapidly by cytokine treatment than VCAM-1 and ICAM-1 (for a review, see reference 26) because its promoter is capable of recruiting three (if all three sites are bound by the p50/p65 heterodimer) or even four (if site 1 is occupied by a p65 homodimer) p65 subunits. p65, in contrast to p50, harbors a transcriptional activation domain (for a review, see reference 16). Therefore, the very rapid onset of cytokine-induced E-selectin transcription may be determined by the simple recruitment of multiple p65 subunits to the promoter.

NF-κB binding sites 1 and 2 are conserved through evolution. The human and murine E-selectin promoters share a high degree of sequence similarity. However, the murine DNA sequence (GaGaATTTCC) differs by 2 bp (lowercase letters) from the previously identified human NF-κB site 3 sequence (GGGGATTTCC). In addition, it has been shown that the murine DNA element is not recognized by NF-κB (2), despite the fact that the promoter is highly responsive to cytokines. These seemingly paradoxical observations may now be explained by the identification of two additional NF-κB sites in the human E-selectin promoter. A sequence comparison between the human (GTGGATATTCCCGGGAAAGTTT) and murine (GTGGATATTCCCaGaAAAcTTT) promoters in the region encompassing NF-κB sites 1 and 2 reveals only a 3-bp difference (lowercase letters). Furthermore, we have demonstrated herein that the murine promoter element is bound by NF-κB, suggesting that the cytokine-induced expression of the E-selectin gene in mouse requires the DNA sequences homologous to the human NF-κB sites 1 and 2. These findings are consistent with the interpretation that the newly identified regulatory region encompassing NF-κB binding sites 1 and 2 is crucial for the TNF-α-induced expression of the E-selectin gene.

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REFERENCES

1. **Baeuerle, P. A., and T. Henkel.** 1994. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. **12:**141–179.
2. **Becker-Andre, M., R. Hooft van Huijsduijnen, C. Losberger, J. Whelan, and J. F. DeLamarter.** 1992. Murine endothelial leukocyte-adhesion molecule 1 is a close structural and functional homologue of the human protein. Eur. J. Biochem. **206:**401–411.
3. **Beg, A. A., and A. S. Baldwin, Jr.** 1993. The IkB proteins: multifunctional regulators of Rel/NF-κB transcription factors. Genes Dev. **7:**2064–2070.
4. **Beg, A. A., T. S. Finco, P. V. Nantermet, and A. S. Baldwin, Jr.** 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. **13:**3301–3310.
5. **Bevilacqua, M. P., S. Stengelin, M. A. Gimbrone, Jr., and B. Seed.** 1989. Endothelial leukocyte adhesion molecule 1: an inducible

receptor for neutrophils related to complement regulatory proteins and lectins. Science **243:**1160–1165.
6. **Blank, V., P. Kourilsky, and A. Israel.** 1992. NF-κB and related proteins: Rel/dorsal homologies meet ankyrin-like repeats. Trends Biochem. Sci. **17:**135–140.
7. **Butcher, E. C.** 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell **67:**1033–1036.
8. **Collins, T., H. J. Palmer, M. Z. Whitley, A. S. Neish, and A. J. Williams.** 1993. A common theme in endothelial activation. Insights from the structural analysis of the genes for E-selectin and VCAM-1. Trends Cardiovasc. Med. **3:**92–97.
9. **Collins, T., A. Williams, G. I. Johnston, J. Kim, R. Eddy, T. Shows, M. A. Gimbrone, Jr., and M. P. Bevilacqa.** 1991. Structure and chromosomal location of the gene for endothelial-leukocyte adhesion molecule 1. J. Biol. Chem. **266:**2466–2473.
10. **Cotran, R. S., M. A. Gimbrone, Jr., M. P. Bevilacqua, D. L. Mendrick, and J. S. Pober.** 1986. Induction and detection of a human endothelial activation antigen in vivo. J. Exp. Med. **164:** 661–668.
11. **Du, W., D. Thanos, and T. Maniatis.** 1993. Mechanisms of transcriptional synergism between distinct virus-inducible enhancer elements. Cell **74:**887–898.
12. **Elgin, S. C. R.** 1988. The formation and function of DNase I hypersensitive sites in the process of gene activation. J. Biol. Chem. **263:**19259–19262.
13. **Enver, T., A. C. Brewer, and R. K. Patient.** 1985. Simian virus 40 mediated *cis* induction of the Xenopus β-globin DNaseI hypersensitive site. Nature (London) **318:**680–683.
14. **Ghersa, P., R. Hooft van Huijsduijnen, J. Whelan, and J. F. DeLamarter.** 1992. Labile proteins play a dual role in the control of endothelial leukocyte adhesion molecule-1 (ELAM-1) gene regulation. J. Biol. Chem. **267:**19226–19232.
15. **Gilmore, T. D., and P. J. Morin.** 1993. The IkB proteins: members of a multifunctional family. Trends Genet. **9:**427–433.
16. **Grilli, M., J. S. Chiu, and M. J. Lenardo.** 1993. NF-κB and Rel—participants in a multiform transcriptional regulatory system. Int. Rev. Cytol. **143:**1–63.
17. **Gross, D. S., and W. T. Garrard.** 1988. Nuclease hypersensitive sites in chromatin. Annu. Rev. Biochem. **57:**159–197.
18. **Hai, T., F. Liu, W. J. Coukos, and M. R. Green.** 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. **3:**2083–2090.
19. **Hooft van Huijsduijnen, R., J. Whelan, R. Pescini, M. Becker-Andre, A.-M. Schenk, and J. F. DeLamarter.** 1992. A T-cell enhancer cooperates with NF-κB to yield cytokine induction of E-selectin gene transcription in endothelial cells. J. Biol. Chem. **267:**22385–22391.
19a. **Hou, J., V. Baichwal, and Z. Cao.** Proc. Natl. Acad. Sci. USA, in press.
20. **Iademarco, M. F., J. J. McQuillan, G. D. Rosen, and D. C. Dean.** 1992. Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1) J. Biol. Chem. **267:**16323–16329.
21. **Karim, F. D., L. D. Urness, C. S. Thummel, M. J. Klemsz, S. R. McKercher, A. Celada, C. Van Beveren, R. A. Maki, C. V. Gunther, J. A. Nye, and B. J. Graves.** 1990. The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. Genes Dev. **4:**1451–1453.
22. **Kaszubska, W., R. Hooft van Huijsduijnen, P. Ghersa, A.-M. DeRaemy-Schenk, B. P. C. Chen, T. Hai, J. F. DeLamarter, and J. Whelan.** 1993. Cyclic AMP-independent ATF family members interact with NF-κB and function in the activation of the E-selectin promoter in response to cytokines. Mol. Cell. Biol. **13:**7180–7190.
23. **Kunsch, C., S. M. Ruben, and C. A. Rosen.** 1992. Selection of optimal kB/Rel DNA-binding motifs: interaction of both subunits of NF-κB with DNA is required for transcriptional activation. Mol. Cell. Biol. **12:**4412–4421.
24. **Liou, H.-C., and D. Baltimore.** 1993. Regulation of the NF-κB/rel transcription factor and IkB inhibitor system. Curr. Opin. Cell Biol. **5:**477–487.
25. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
26. **McEver, R. P.** 1991. Selectins: novel receptors that mediate leukocyte adhesion during inflammation. Thromb. Haemostasis **65:**223–228.
27. **Montgomery, K. F., L. Osborn, C. Hession, R. Tizard, D. Goff, C. Vassallo, P. I. Tarr, K. Bomsztyk, R. Lobb, J. M. Harlan, and T. H. Pohlman.** 1991. Activation of endothelial-leukocyte adhesion molecule 1 (ELAM-1) gene transcription. Proc. Natl. Acad. Sci. USA **88:**6523–6527.
28. **Narayanan, R., K. A. Higgins, J. R. Perez, T. A. Coleman, and C. A. Rosen.** 1993. Evidence for differential functions of the p50 and p65 subunits of NF-κB with a cell adhesion model. Mol. Cell. Biol. **13:**3802–3810.
29. **Neish, A. S., A. J. Williams, H. J. Palmer, M. Z. Whitley, and T. Collins.** 1992. Functional analysis of the human vascular cell adhesion molecule 1 promoter. J. Exp. Med. **176:**1583–1593.
30. **Osborn, L.** 1990. Leukocyte adhesion to endothelium in inflammation. Cell **62:**3–6.
31. **Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, and R. Lobb.** 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell **59:**1203.
32. **Osborn, L., S. Kunkel, and G. J. Nabel.** 1989. Tumor necrosis factor a and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. Proc. Natl. Acad. Sci. USA **86:**2336–2340.
33. **Perkin, N. D., R. M. Schmid, C. S. Duckett, K. Leung, N. R. Rice, and G. J. Nabel.** 1992. Distinct combinations of NF-κB subunits determine the specificity of transcriptional activation. Proc. Natl. Acad. Sci. USA **89:**1529–1533.
34. **Pober, J. S., M. A. Gimbrone, Jr., L. A. Lapierre, D. L. Mendrick, W. Fiers, R. Rothlein, and T. A. Springer.** 1986. Overlapping patterns of activation of human endothelial cells by interleukin-1, tumor necrosis factor, and immune interferon. J. Immunol. **137:** 1893.
34a. **Schindler, U., and V. R. Baichwal.** Unpublished data.
35. **Shu, H. B., A. B. Agranoff, E. G. Nabel, K. Leung, C. S. Duckett, A. S. Neish, T. Collins, and G. J. Nabel.** 1993. Differential regulation of vascular cell adhesion molecule 1 gene expression by specific NF-κB subunits in endothelial and epithelial cells. Mol. Cell. Biol. **13:**6283–6289.
36. **Thompson, C. C., T. A. Brown, and S. L. McKnight.** 1991. Convergence of Ets- and Notch-related structural motifs in a heteromeric DNA binding complex. Science **253:**762–768.
37. **Tjian, R., and T. Maniatis.** 1994. Transcriptional activation: a complex puzzle with few easy pieces. Cell **77:**5–8.
38. **Whelan, J., P. Ghersa, R. Hooft van Huijsduijnen, J. Gray, G. Chandra, F. Talabot, and J. F. DeLamarter.** 1991. An NF-κB-like factor is essential but not sufficient for cytokine induction of endothelial leukocyte adhesion molecule 1 (ELAM-1) gene transcription. Nucleic Acids Res. **19:**2645–2663.