An NFκB-like factor is essential but not sufficient for cytokine induction of endothelial leukocyte adhesion molecule 1 (ELAM-1) gene transcription

James Whelan, Paola Ghersa, Rob Hooft van Huijsduijnen, John Gray[1], Gyan Chandra[1], François Talabot and John F.DeLamarter
Glaxo Institute for Molecular Biology, 14 chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland and [1]Glaxo Inc., North Carolina, USA

Received February 21, 1991; Revised and Accepted April 29, 1991

ABSTRACT

The endothelial leukocyte adhesion molecule 1 (ELAM-1) is transiently expressed specifically on the surface of cytokine-induced endothelial cells. We demonstrate that the transient expression of the protein is paralleled by an increase and decrease in transcription of the ELAM-1 gene. To identify the cis-acting transcription control regions within the ELAM-1 gene that are responsible for this cytokine-induced expression, we isolated and analyzed an ELAM-1 genomic clone containing sequences upstream of the transcription start site. We constructed a series of ELAM-1 deletion mutants linked to a reporter gene and analyzed their expression in both endothelial and non-endothelial cells. Results show that a fragment of 233 bp upstream of the transcription start site is sufficient to confer cytokine inducibility upon the reporter gene in both endothelial and non-endothelial cells. Further analysis defined two elements within this region that are involved in the cytokine inducibility of the ELAM-1 gene. One element lies within the −233 to −117 region, the other element represents an NFκB consensus binding site between nucleotides −94 to −85. Gel shift analysis reveals increased binding of an NFκB-like factor to this consensus sequence in extracts prepared from IL-1-induced endothelial cells. The results suggest that cytokine induction of ELAM-1 gene transcription is imparted by a combination of positive factors, one being an NFκB-like transcription factor, interacting with cis-acting elements within the enhancer/promoter of the gene.

INTRODUCTION

One of the first steps in an inflammatory or immune response at the site of injury is the increased adhesion of leukocytes to the endothelium. This involves the increased expression of adhesion molecules on the endothelial cell surface and their corresponding receptors on the surface of leukocytes. The increased expression of adhesion molecules and their receptors is mediated by the release of various inflammatory mediators (e.g. cytokines). Abnormal expression of these adhesion molecules has also been implicated in a number of acute and chronic inflammatory diseases (1). Endothelial leukocyte adhesion molecule 1 (ELAM-1) represents one such adhesion molecule which is rapidly expressed on the surface of cytokine-induced endothelial cells (2).

ELAM-1 is a member of a family of structurally related adhesion proteins referred to as selectins (3). The other members of this family include the lymphocyte homing receptor, Mel-14 (LAM-1) and PADGEM (GMP140) which is expressed on both platelets and endothelium. They are all composed of an N-terminal lectin domain, followed by an EGF-homologous domain, a number of complement consensus repeats which varies between the different members of the family, a transmembrane sequence and a short cytoplasmic tail. The complement consensus repeats have been hypothesized to serve to position the N-terminal lectin-like putative binding sites at varying distances from the plasma membrane (4). This family of proteins play a role in leukocyte-endothelial adhesion (1). ELAM-1 has been shown to be involved in neutrophil adhesion to endothelium *in vitro* (2). The fact that ELAM-1 is rapidly expressed on the endothelial cell surface, following cytokine induction, has led to the suggestion that ELAM-1 may function as an early step in inflammatory cytokine-induced adhesion of neutrophils to endothelium. Thus ELAM-1 may have an important role in acute inflammation and the disorders associated with it (for reviews, see 1, 4).

ELAM-1 protein expression appears to be restricted to the surface of induced endothelial cells (2). Cytokine-induced expression of ELAM-1 on the endothelial cell surface is paralleled by an increase in ELAM-1 mRNA (3). Cytokine induction likely involves increased ELAM-1 transcription initiation, increased mRNA stability or a combination of both.

With these observations in mind we undertook to examine the mechanisms involved in the control of ELAM-1 expression. Our studies have been aimed at investigating the transcription regulation mechanisms of the ELAM-1 gene, in particular the mechanism involved in cytokine induction of the gene. As has been demonstrated for many eukaryotic genes, transcription initiation is regulated by the interaction of multiple transacting factors with cis-acting elements predominantly in the 5'-flanking

region of the gene (for review, see 5, 6). In addition, the importance of protein-protein interactions between transcription factors is becoming increasingly evident (for reviews, see 7, 8). The variety of DNA binding sites and transcription factors that interact with them suggests that regulation of gene transcription results from interactions between multiple trans-acting factors.

To investigate the mechanisms involved in cytokine-induced expression of ELAM-1, we first established that this induction was primarily at the level of increased transcription initiation. Isolation of a genomic DNA clone spanning the human ELAM-1 gene and determination of the transcription start site allowed us to investigate cis-acting elements within the promoter/enhancer region of the gene involved in cytokine-induced transcription initiation. We have defined two regions upstream of the ELAM-1 gene transcription start site which are involved in cytokine-induced expression of the gene. One of these cis-acting elements is an NFκB binding consensus sequence and cytokine induction results in increased binding of an NFκB-like factor to this site. Our data indicate that neither element alone is sufficient to confer cytokine inducibility upon the gene but rather that cytokine induction involves a concomitant interaction between an NFκB-like factor and an as yet unidentified transcription factor(s).

MATERIALS AND METHODS

Cell lines

Human umbilical vein endothelial cells (HUVEC) were extracted from human umbilical cords by collagenase treatment. The cells were cultured in medium MCDB 131 supplemented with epidermal growth factor (10 ng/ml), hydrocortisone (1 ng/ml), bovine brain extract containing heparin, 2% fetal bovine serum, gentamicin and amphotericin (Clonetics, CA). IE7 cells are simian virus 40 (SV40) transformed primary HUVECs (Dix *et al.*, manuscript in preparation) and were grown in the endothelial growth medium described above, containing geneticin (300 μg/ml). HeLa cells were grown in Dulbecco modified Eagle medium containing 10% (vol/vol) fetal calf serum and 50 μg each of penicillin and streptomycin per ml.

Nuclear run-on assay

HUVEC were treated with human IL-1β (10 U/ml) and nuclei isolated at different time points following induction. Nuclear run-on assays were preformed as previously described (9) using 3×10^7 nuclei for each point. RNA transcripts were isolated by the guanidium thiocyanate procedure (10). Approximately 10 c.p.m. of each [^{32}P] RNA run-on product was hybridized to DNA (5 μg each) applied onto nitrocellulose filters. The DNA probes were the plasmid pCDM8, pELAM-1 (plasmid pCDM8 containing ELAM-1 cDNA) and a plasmid containing von Willebrand factor cDNA in the plasmid pBG312. Results were visualized by autoradiography and quantitated by densitometric scanning of the autoradiogram.

Isolation of an ELAM-1 genomic clone

A human genomic library constructed in the modified lambda phage EMBL3 (#HL1006d from Clontech laboratories, Palo Alto, CA) was screened for ELAM-1 sequences using oligonucleotide probes complementary to the ELAM-1 cDNA (3). A phage which repeatedly hybridized to these oligonucleotides was purified and shown to contain an insertion of approximately 17 kb. From this phage a 1558 bp *Hind*III to *Sal*I fragment which hybridized to these probes was subcloned into pUC for further manipulations.

Primer extension analysis

Thirty μg of total RNA was hybridized with a 5′ end-labeled 36 mer oligonucleotide, 3′TGGACTCTGTCTCCGTCGTCA-CTATGGGTGGACTCT-5′, complementary to bases +41 to +76 of the non-coding strand of the human ELAM-1 gene. Analysis was carried out as indicated by Ansubel *et al.* (11). The final product was analyzed on a 8% polyacrylamide sequencing gel and visualized by autoradiography.

Plasmid constructions

To construct 5′-flanking deletion mutants of the human ELAM-1 gene, *Bst*YI sites present both immediately 5′ to the ELAM-1 fragment and 80 nucleotides 3′ to the transcription start site together with restriction enzyme sites within the 5′-flanking region of the gene were used to isolate the desired fragments. These fragments were then subcloned into a chloramphenicol acetyltransferase (CAT) expression vector, pCAT-Basic (Promega, Madison, WI). The 5′-flanking restriction enzyme sites, *Bst*YI, *Acc*I, *Nsi*I and *Sma*I were used to generate the −741, −383, −233 and −117 chimeras respectively. The deletion mutant with an end point at −35 was generated using two oligonucleotides, 5′GCGCGAAGCTTCCTCCTATAAAAGG 3′ and 5′GCGCGTCTAGATCTCAGGTGGGTAT 3′, a portion of each being complementary to −35 to −22 of the coding strand and +63 to +76 of the non-coding strand respectively. These oligonucleotides were then used in the polymerase chain reaction to amplify the intervening sequence (−35 to +76). The sequence of these oligonucleotides was such that *Hind*III and *Xba*I sites were generated at the 5′ and 3′ ends, respectively, of the amplified fragment. This fragment was subcloned into pCAT-Basic to generate the −35 chimera. The −233 to −117 internal deletion mutants were generated using the *Nsi*I and *Sma*I restriction sites, removal of the intervening ELAM-1 fragment and re-ligation. The −383 (NFκB mutant) was generated by oligonucleotide directed *in vitro* mutagenesis as described by Amersham.

DNA transfection and CAT analysis

Approximately 12 h before transfection IE7 and HeLa cells were plated at densities of 1×10^6 per 60 mm plate and 0.5×10^5 per 100 mm plate respectively. Transfection of plasmid DNA into IE7 cells was carried out by the DEAE-Dextran method as described by Cullen (12). Transfection of plasmid DNA into HeLa cells was carried out by the calcium phosphate co-precipitation method as described previously (13). Each experiment was repeated several times with at least two different plasmid preparations. CAT assays were carried out essentially as described by Gorman *et al.* (14). CAT enzymatic activity was quantitated by scintillation counting of spots excised from the chromatograms.

Nuclear extract preparation and gel shift analysis

HUVEC (passage 5−6) nuclear extracts were prepared as described by Dignam *et al.* (15) with the modifications described by Dorn *et al.* (16). Protein concentrations were adjusted to 3 μg/μl prior to aliquoting and stored at −70°C. Oligonucleotides containing the following sequences were synthesized: an oligonucleotide containing the human immunoglobulin κ light

chain NFκB consensus sequence (5'-AAATAAAAAGGGGG-ATTTCCAAAAAATAAA-3'), and a corresponding oligonucleotide with a mutated NFκB consensus sequence (5'-A-AATAAAAAGGGAAAAATTCAAAAAATAAA-3'). An oligonucleotide containing the ELAM-1 NFκB consensus sequence (5'-AAAAAAAATTGGGGATTTCCTCAAAAAA-AA-3'), and a corresponding oligonucleotide with a mutated ELAM-1 NFκB consensus sequence (5'-GGATGCCATTCCC-CTAAACCTCTTTACTGG-3'). The CCAAT-box oligonucleotide is derived from the murine MHC class II Eα gene, ATTTT-TCTGATTGGTTAAAAGT (16), these oligonucleotides were 5' end-labeled. Binding reactions contained 50 mM HEPES (pH 7.9); 1 mM dithiothreitol; 1 mg/ml bovine serum albumin; 50 mM KCl; 10 mM MgCl; 0.125 μg/μl of poly (dI-dC); 30% (vol/vol) glycerol and 6 μg of extract protein. Each 20 μl reaction was incubated at room temperature for 10 min. Approximately 6000 c.p.m of ^{32}P-labeled oligonucleotide was then added to the reaction and incubated for a further 10 min at room temperature. Samples were then subjected to electrophoretic separation on a 4.5% non-denaturing polyacrylamide gel. Following electrophoresis, gels were dried and labeled DNA was localized by autoradiography.

RESULTS

Cytokine treatment of HUVEC cells results in transcriptional regulation of the ELAM-1 gene

To determine whether regulation of ELAM-1 expression was mediated by changes in transcription of the gene, we performed nuclear run-on experiments. Nuclei were isolated from untreated endothelial cells and at various time points following IL-1 treatment of endothelial cells. Radiolabeled RNA generated by these nuclear preparations was hybridized to ELAM-1 cDNA in plasmid pCDM8, von Willebrand sequences in pBG312, and the pCDM8 plasmid alone. No hybridization was seen between the RNA and the pCDM8 plasmid alone. Equal amounts of RNA from all samples hybridized to an equal extent with the von Willebrand probe (data not shown). In contrast, hybridization to the ELAM-1 cDNA probe varied significantly, indicating changes in transcription initiation. The transcriptional activity of ELAM-1 gene was not detectable in uninduced HUVEC cells. There was a rapid increase in ELAM-1 gene transcription initiation within 30 minutes of IL-1 treatment, reaching a maximal level 2–4 hours after IL-1 treatment. The rate of transcription initiation returned to near-basal level activity 24 h following IL-1 treatment (Figure 1).

From these results we conclude that IL-1 induces a rapid increase in ELAM-1 gene transcription initiation which is followed by a rapid decrease (downregulation) in the rate of transcription initiation. This induction/downregulation of transcription initiation parallels the pattern of ELAM-1 protein expression on the cell surface, following IL-1 treatment, as determined by FACS analysis (Figure 1, see insert). We conclude that the rapid changes in ELAM-1 protein expression on HUVEC cells results, at least in part, from changes in the rate of transcription initiation of the ELAM-1 gene.

Organization of the ELAM-1 promoter/enhancer

To analyze the promoter/enhancer region of the ELAM-1 gene, genomic DNA sequence which precedes the cDNA encoding sequences was sought. A library of human genomic fragments in a lambda phage vector was screened using oligonucleotide probes complementary to sequences in the 5' end of the cDNA (see Materials and Methods). From a probe-positive recombinant phage, a fragment of 1.5 kb containing the extreme 5' end of the cDNA sequence was isolated for further analysis. Following nucleotide sequencing, the fragment was found to harbor several interesting features (Figure 2). The sequence, as expected, contained the most 5' portion of the published cDNA sequence for human ELAM-1 (3). Unexpectedly, two introns were found to interrupt the first 200 nucleotides of the cDNA breaking the untranslated 5' region in two and interrupting the coding region 37 nucleotides after the putative translation start signal.

The genomic sequence preceding the start of the cDNA sequence revealed several features of a promoter/enhancer region. CAAT and TATA box sequences were identified in the expected localizations suggesting a minimal promoter organization. Moreover, a second motif corresponding to a consensus sequence for the NFκB binding sequence (17) was found to start 27 nucleotides upstream of the CAAT box. Sequences with partial homology to the binding sites of other known factors could be found further upstream in the fragment. A large partially palindromic sequence, representing a potential DNA binding motif, was found 119 nucleotides upstream of the NFκB binding sequence (Figure 2).

Since the ELAM-1 gene contains a perfect TATA sequence, we speculated that a single transcription start site would exist approximately 30 bp downstream (18). To identify the RNA start site, we mapped the start of transcription. Primer extension analysis using an oligonucleotide complementary to a region downstream of the predicted start site was performed (Figure 3). RNA isolated from uninduced HUVEC cells did not result in an extended product (Figure 3, lane 1). This was expected

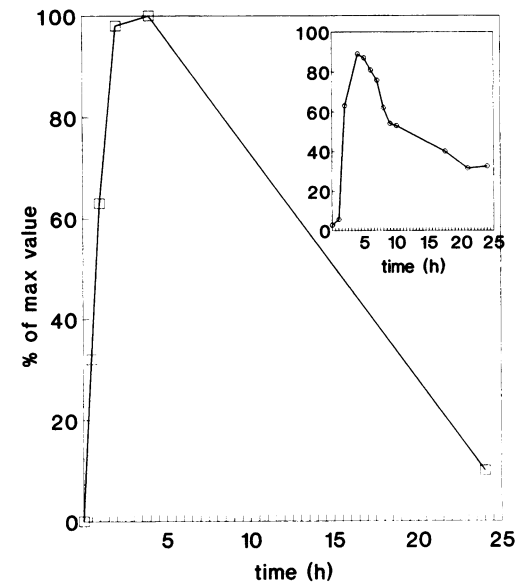

Figure 1. Nuclear run-on analysis of ELAM-1 gene expression at different time points following IL-1 induction. Nuclei were isolated from both uninduced and IL-1β (10 U/ml) induced HUVECs, followed by preparation of ^{32}P-labeled transcripts which were hybridized to a plasmid containing ELAM-1 cDNA immobilized on nitrocellulose. The results were quantitated by densitometric scanning of the resulting autoradiogram and are plotted as a percentage of maximal density. Insert: ELAM-1 expression on the HUVEC surface (plotted as percentage of positive cells) at various time points following IL-1β (10 U/ml) induction as determined by indirect immunofluorescence and quantitation using a Becton Dickinson FACS analyzer.

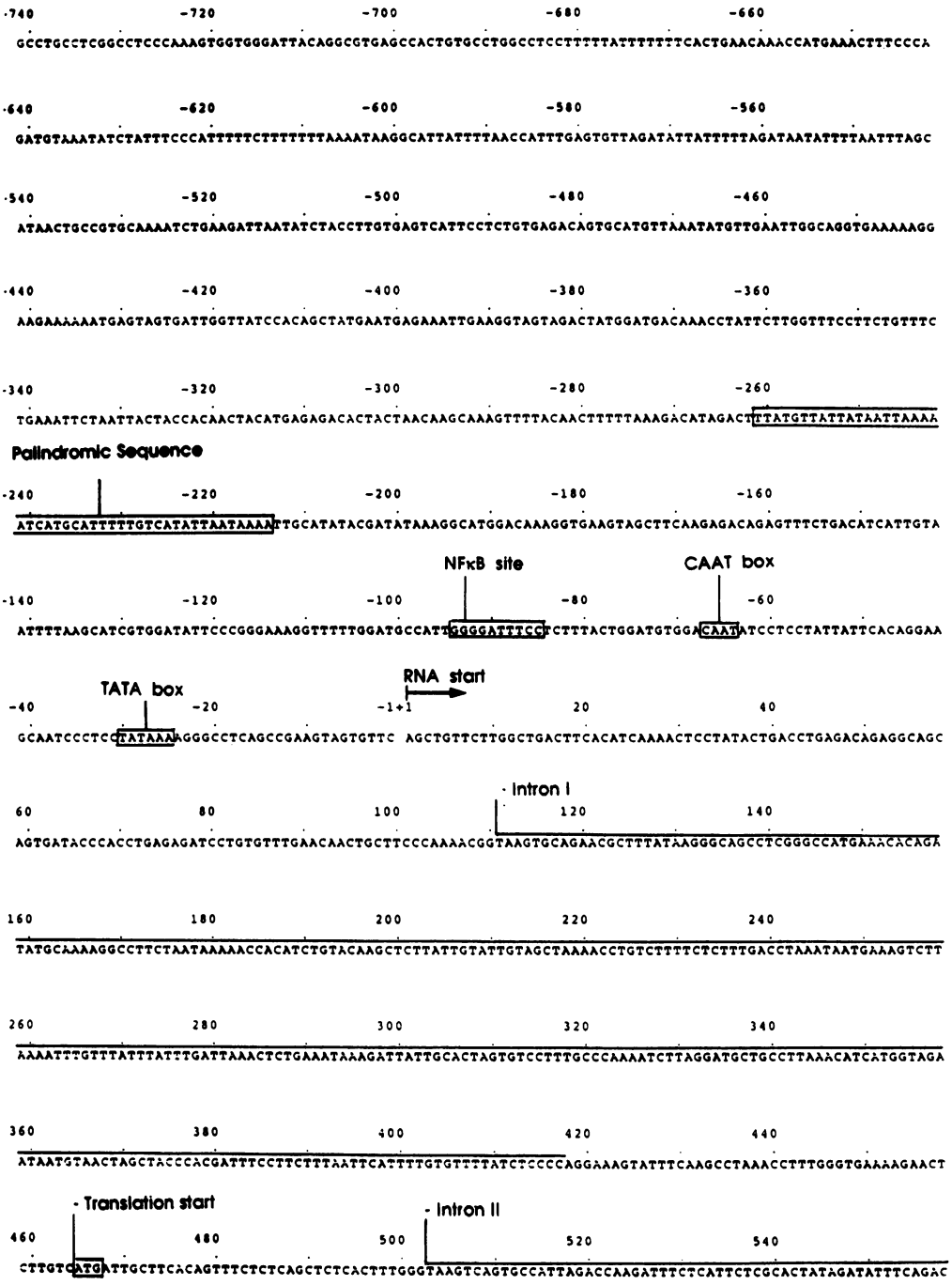

Figure 2. Nucleotide sequence of the promoter/enhancer region of human ELAM-1. The nucleotide sequence is numbered according to the initiation of transcription with sequence motifs identified by boxes. Both strands of the DNA were sequenced in overlapping fragments by the dideoxy-chain termination method.

since ELAM-1 is not expressed in uninduced HUVEC cells. Analysis of RNA isolated from either IL-1 or TNF-induced HUVEC cells resulted in an extended product of 76 nucleotides (lanes 2 and 3 respectively). Thus, transcription is initiated at a single site 30 bp downstream of the TATA box at an adenine residue identified in Figure 2 as position +1.

Identification of 5′-flanking sequences that regulate ELAM-1 gene expression

As a step towards identifying the 5′-flanking sequences necessary for cytokine-induced ELAM-1 expression, we subcloned a series of 5′-flanking deletion mutants into a bacterial CAT expression vector (Figure 4). The CAT activity expressed by these chimeras was assayed in a transformed HUVEC line (IE7 cells, Dix *et al.*, manuscript in preparation) and HUVECs 40 to 48 h after transfection.

All the 5′-flanking deletion mutant plasmids depicted in figure 4, except the 5′-deletion mutant extending 35 nucleotides upstream of the transcription start site, had a very low level of basal activity in uninduced IE7 cells (Figure 5A). The −35 5′-deletion mutant is inactive in both uninduced and IL-1-induced IE7 cells as one would expect for a chimera which contains only

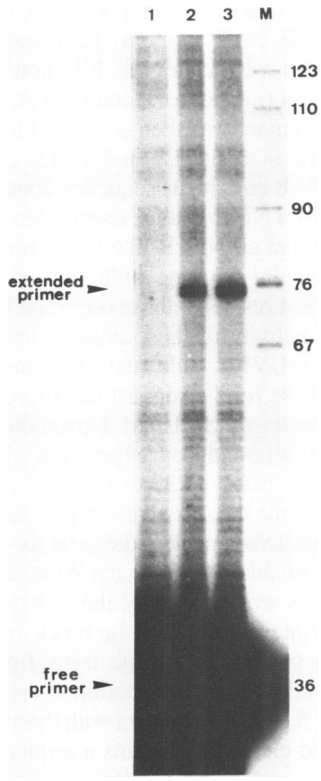

Figure 3. Analysis of transcription start site by primer extension analysis. Total RNA was isolated from uninduced, lane 1; IL-1β (10 U/ml) induced, lane 2 and TNFa (200 U/ml) induced, lane 3; HUVECs. Primer extension analysis was carried out as described in Materials and Methods. The right lane (M) represents the size marker, end-labeled *Msp*I cut pBR322.

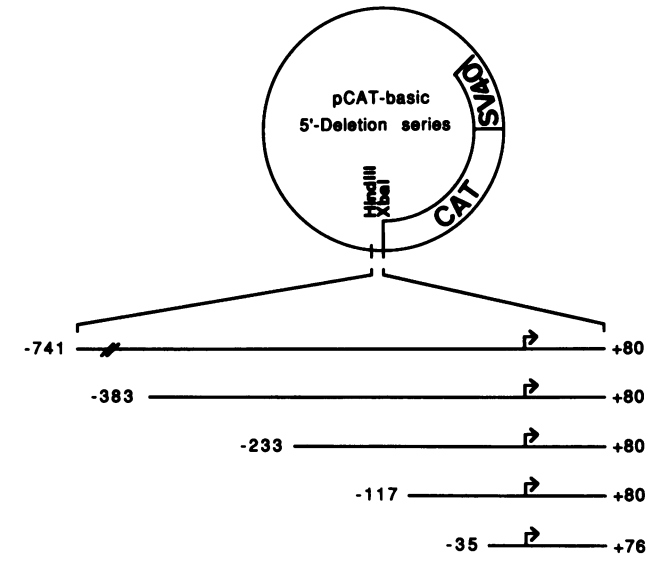

Figure 4. Construction of the ELAM-1 gene-CAT 5'-deletion mutant vectors. The vectors were constructed as described in Materials and Methods. A schematic view of the vector and the ELAM-1 gene 5'-flanking sequences used in these constructs is depicted.

the TATA box and no other upstream sequences. The deletion mutants with end points at −741, −383, and −233 nucleotides upstream of the transcription start site all show approximately equivalent levels of induced activity following IL-1 treatment of cells. However, the deletion mutant with 117 nucleotides of upstream sequence has a very low level of IL-1-induced activity (Figure 5A). This suggests that a critical element(s) involved in IL-1 induction lies within the −233 to −117 region. The levels of activity observed from these chimeras, following TNF induction, were very similar to those observed with IL-1 (data not shown). An essentially identical expression pattern has also been demonstrated in primary HUVEC. In addition, RNase protection analysis confirmed that transcription initiation is from the correct ELAM-1 transcription start site (data not shown).

ELAM-1 is expressed uniquely on endothelial cells (2). Transcription directed by the ELAM-1 promoter/enhancer therefore should be restricted to this cell type. The transformed human epithelial cells, HeLa cells, were used as an example of non-endothelial cells for testing cell type specificity. We first determined that ELAM-1 mRNA is not detectable in either uninduced or cytokine-induced HeLa cells by Northern analysis (data not shown). Using the chimeras described above, we determined whether these ELAM-1 promoter/enhancer sequences were able to restrict reporter gene expression to endothelial cells. We assayed the reporter gene activity, regulated by the 5'-deletion mutants of the ELAM-1 promoter/enhancer, in uninduced and IL-1-induced HeLa cells. The highest levels of reporter gene activity were observed in HeLa cells of any cell type tested. This most likely reflects a significantly higher transfection efficiency of HeLa cells, as determined by comparing the activity of an SV40-driven control plasmid transfected into the different cell types. The pattern of expression of this series of 5'-deletion mutants in HeLa cells, following IL-1 induction, reflects that seen in both IE7 and HUVEC cells (Figure 5B). These results suggest at least part of the mechanism involved in cytokine induction of ELAM-1 in endothelial cells is functional in this non-endothelial cell type. The element(s) involved in conferring cell specificity upon the gene would therefore appear to lie outside −741 to +80 region of the ELAM-1 gene.

Two separate cis-acting elements are involved in cytokine induction of ELAM-1 gene transcription.

The data presented above demonstrated that cytokine-induced expression of the ELAM-1 gene is regulated by 5'-flanking sequences lying between nucleotides −233 to −117. In addition, an NFκB consensus binding sequence can be found between nucleotides −94 to −85 upstream of the ELAM-1 transcription start site. The NFκB transcription factor is believed to play a role in cytokine induction of certain genes (19 and 20). To further examine the role of both these elements in cytokine-induced expression of the ELAM-1 gene, we constructed a series of internal and block mutations within these regions.

Internal deletion mutants of the −233 to −117 region were constructed in both the −741 and −383 CAT chimeras. The CAT enzymatic activities of these mutants, in both uninduced and IL-1-induced, transfected IE7 and HeLa cells were compared to the activity of the wild type chimeras. The internal deletion mutants demonstrated no IL-1-inducible activity in either IE7 or HeLa cells (Figure 6). These results demonstrate the importance of this region in control of cytokine induction of the ELAM-1 gene. Since no known transcription factor binding site can be found within this region, we conclude that an as yet unidentified cytokine-responsive enhancer element may be present.

To determine whether the NFκB consensus binding site plays a role in cytokine-induced expression, we constructed a block

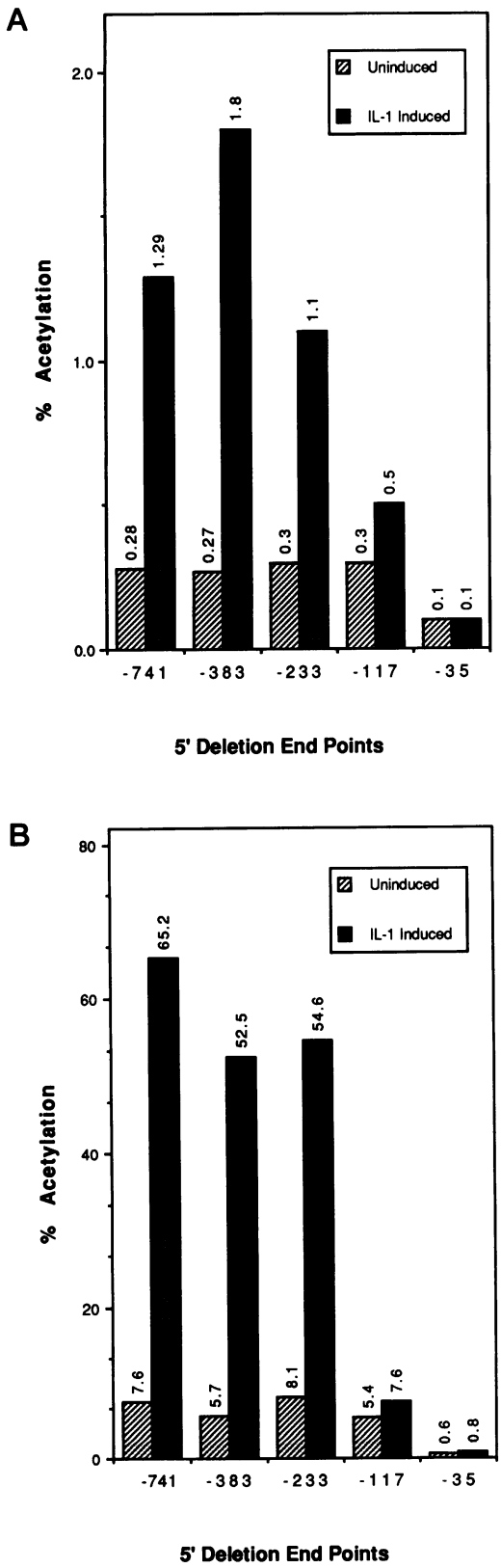

Figure 5. CAT enzymatic activity of 5'-flanking ELAM-1 deletion mutants in uninduced or IL-1β (10 U/ml) induced IE7 cells (A) and in HeLa cells (B). IE7 and HeLa cells were transfected as described in the Materials and Methods, using 10 μg of test plasmid DNA. Reporter gene activity was assayed by measuring CAT enzyme levels. The results were reproduced in at least three separate experiments; data shown are from one representative experiment.

mutation of the homologous ELAM-1 sequence (nucleotides −94 to −85) in the −383 CAT chimera. Each base between −94 to −85 inclusive was changed by non-complementary transversion mutation (i.e., G to T and C to A and vice versa). The activity of this mutant was then compared to that of the wild type −383 chimera in both IE7 and HeLa cells (Figure 6). Mutation of the NFκB consensus sequence completely inhibited the ability of IL-1 or TNF to induce expression of this chimera. These data suggest that an NFκB-like factor binding to this site plays a critical role in cytokine induction of ELAM-1 gene transcription. Identical results to those observed in IE7 cells were obtained when the activity of these internal and block mutants were examined in HUVEC cells (data not shown).

Taken together these results suggest that there are at least two separate elements within the ELAM-1 promoter/enhancer that are involved in cytokine-induced expression of the gene. One element lies within the −233 to −117 region of the promoter/enhancer while the second element is the NFκB binding consensus sequence between nucleotides −94 to −85. Separately, these elements are unable to confer cytokine inducibility upon the reporter gene, as was shown by the independent mutation of either element (internal deletion or block mutation). Thus, cytokine induction of ELAM-1 gene transcription appears to involve interaction between a combination of cis-acting elements and the trans-acting factors that interact with these elements. Since one of the identified elements contains a sequence homologous to an NFκB consensus binding sequence, we speculate that an NFκB-like factor is one of these important trans-acting factors.

In HUVEC cells IL-1 induces an NFkB-like factor which binds to the ELAM-1 promoter/enhancer region

We wanted to determine if an NFκB (or NFκB-like) protein was able to interact with the ELAM-1 promoter and further whether the binding of such a factor would be cytokine-regulated. To answer these questions, oligonucleotide fragments were

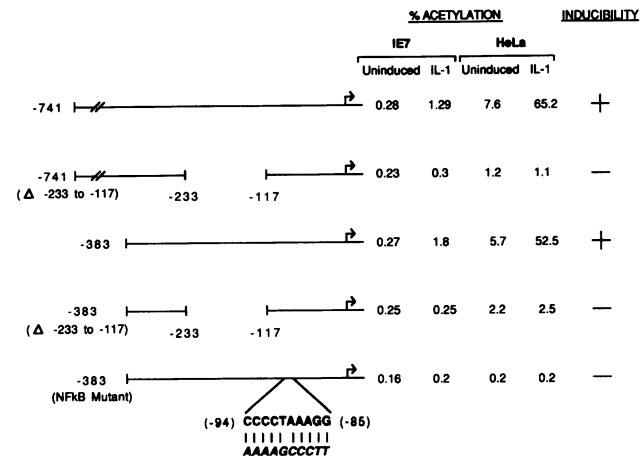

Figure 6. Analysis of the expression of ELAM-1/CAT constructs with internal or block mutations within the ELAM-1 gene 5'-flanking sequences. These mutant constructs were transfected into IE7 and HeLa cells as described in Materials and Methods, using 10 μg of test plasmid DNA. Extracts from transfected cells were prepared and assayed for CAT activity. A schematic representation of the sequences in each of the mutant constructs is shown, along with the ELAM-1 NFκB consensus sequence and the corresponding sequence in the block mutant (below). The percentage acetylation of [^{14}C]-chloramphenicol obtained with extracts prepared from IE7 and HeLa cells that had been transfected with these constructs is shown. The data are representative of at least three experiments.

synthesized. They contained either the core of the human Ig κ enhancer NFκB binding site (κB, 17), the ELAM-1 NFκB consensus binding sequence, with an additional three ELAM-1 nucleotides 5′ and two ELAM-1 nucleotides 3′ to the consensus sequence (ELAM-κB), or NFκB binding site mutant versions of these oligonucleotide fragments (κBmut and ELAM-1mut). The gel mobilities of the wild type labeled DNA fragments were examined following their incubation with nuclear protein extracts prepared from either uninduced or IL-1-induced HUVEC cells (Figure 7A and B respectively). A single protein-DNA specific complex formed with both κB and ELAM-κB probes in extracts prepared from IL-1-induced HUVEC cells (Figure 7B). The second faster migrating complex binding to the ELAM-κB probe is non-specific (see below). A similar, but very weak complex, was also formed in the extracts prepared from uninduced HUVEC cells (Figure 7A). Since the IL-1-induced HUVEC nuclear extracts shifted both κB and ELAM-κB oligonucleotide fragments in larger amounts than the nuclear extracts of uninduced HUVEC, IL-1 appears to induce the binding of these proteins to both NFκB consensus sequence probes. Furthermore, the migration of these complexes is the same with both probes. Since the core NFκB consensus binding sequences in both these probes are identical, these complexes likely contain the same or similar proteins.

Competition with excess unlabeled oligonucleotide fragments, corresponding to either wild type (κB or ELAM-κB) or mutant versions of these NFκB consensus sequence oligonucleotide fragments (κBmut or ELAMκBmut), was carried out to address the specificity of binding to these NFκB consensus sequence binding sites. Specificity of binding was confirmed by the inhibition of complex formation by the excess unlabeled probe sequences but not by the mutant version of the probe. In addition, the ELAM-κB oligonucleotide can compete for binding of the protein(s) which binds to the κB oligonucleotide fragment and vice versa. However, the mutant version of ELAM-κB (ELAMκBmut) cannot compete for binding of the protein(s) to the kB oligonucleotide and vice versa. This again suggests that both these probes bind the same or related protein(s). Interestingly, the complex formed with the κB fragment was not as efficiently competed away by ELAM-κB as was the complex formed with the ELAM-κB by κB. One explanation might be that the NFκB-like factor has a higher affinity for the NFκB core sequence alone than for the ELAM-1 NFκB site, with the additional ELAM-1 nucleotides flanking the NFκB consensus sequence. Alternatively, modified forms of the same protein are binding with different affinities to the two sites. Our experiments are unable to distinguish between these possibilities. The faster migrating band seen in the gels is competed away by all the DNA fragments suggesting that this represents non-specific binding.

As a further control for the equivalence of the nuclear fractions prepared from uninduced and induced HUVEC, the binding of a ubiquitous, non-inducible transcription factor was assayed. NF-Y/CP1 binds the CCAAT box and is present in all cells tested in a constitutive fashion (21). Nuclear extracts from uninduced and IL-1-induced HUVEC showed an equal ability to retard the migration of an oligonucleotide fragment containing a CCAAT box sequence (Figure 7C). These data suggest that the two extracts were equivalent and not preferentially depleted for any particular transcription factors.

Finally, the presence of an NFκB-like factor, in the complex formed with the ELAM NFκB consensus sequence, was confirmed by using a polyclonal antiserum against the precursor of the p50 subunit of NFκB (serum 2, 22). Incubation of HUVEC

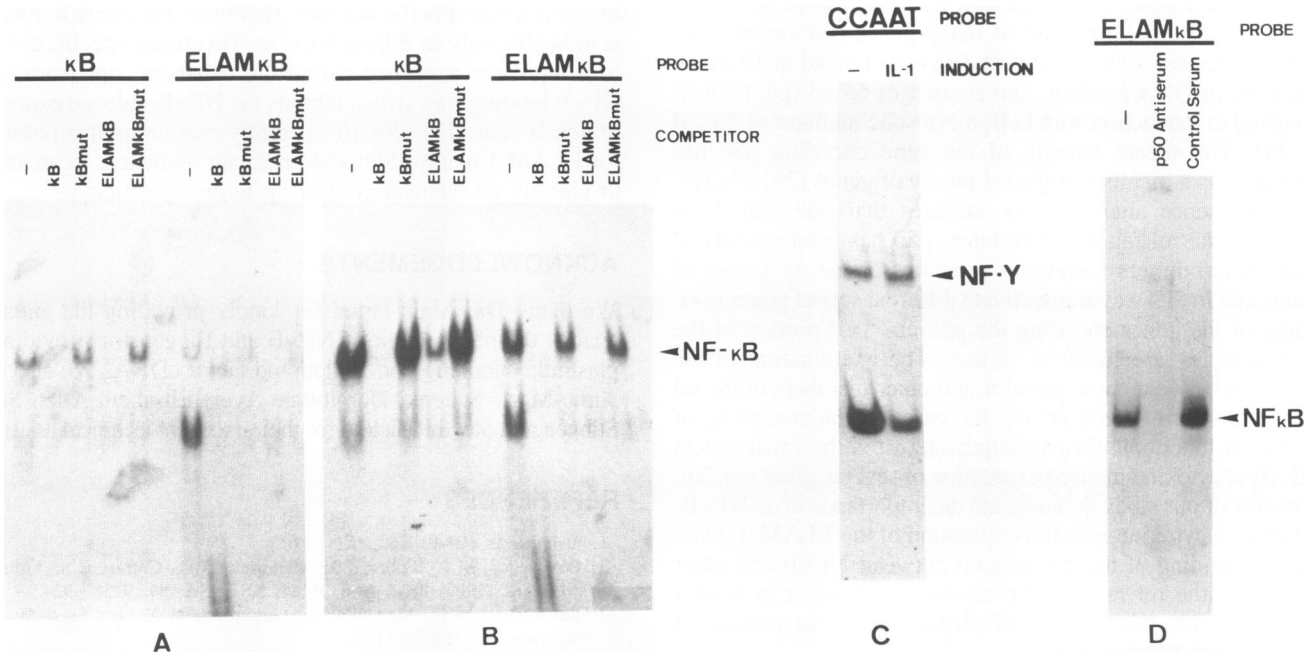

Figure 7. IL-1 induces binding of an NFκB-like factor to the ELAM-1 NFkB consensus sequence in HUVECs. Nuclear extracts prepared from either uninduced (panel A) or IL-1β (10 U/ml) induced (panel B) HUVECs were incubated with labeled oligonucleotide probes (κB and ELAM-κB), present in excess, and the binding of specific nuclear proteins analyzed by gel-shift analysis as described in Materials and Methods. Unlabeled competitor oligonucleotides, present at 1000-fold Molar excess, are indicated above lanes. Panel C shows the binding of the same nuclear extracts to an oligonucleotide fragment containing the sequence CCAAT which binds the constitutively expressed factor NF-Y. Panel D shows the effect of an antibody against the precursor of the p50 subunit of NFκB or control serum on the binding of the nuclear extracts to the ELAMκB probe. The binding reaction was set up as described in Materials and Methods. Prior to the addition of ^{32}P-labeled ELAMκB probe nuclear extracts were preincubated for 30 min at 4°C with either 2 μl of phosphate buffered saline, p50 polyclonal antiserum, or control serum.

nuclear extract with this antiserum inhibited complex formation with the ELAM NFκB consensus sequence as analyzed in the gel shift assay (Figure 7D).

DISCUSSION

The induction and downregulation of ELAM-1 protein expression, following IL-1 treatment, was shown here to correlate with a similar regulation of transcription initiation. An increase in ELAM-1 gene transcription initiation 2.5 h after TNF induction of endothelial cells has been previously demonstrated (23). However, the time course of induction and the correlation of downregulation with transcription arrest was not shown. We have identified and characterized cis-acting elements involved in control of this cytokine-induced expression. At least two elements have been found within the promoter/enhancer region of the ELAM-1 gene which play a critical role in cytokine-induced expression. One element lies within the region between 233 and 117 nucleotides upstream of the transcription start site. Work is currently underway to more accurately identify the control element(s) within this region. Preliminary gel shift analysis reveals a factor binding within this region (unpublished data). The second element involved represents an NFκB binding consensus sequence between nucleotides −94 to −85. Gel shift analysis reveals increased binding of a factor to this element in extracts prepared from IL-1-induced cells. We conclude that this IL-1-induced complex contained an NFκB-like factor for two reasons. First, the complex could be eliminated by competition with wild type (κB) but not the mutant (κBmut) NFκB binding site of the human Ig κ enhancer. Second, formation of the complex was completely inhibited by an antiserum to the p50 subunit of NFκB.

NFκB is a ubiquitously expressed transcription factor which plays a role in the expression of many genes (for review, see 17). NFκB consists of two polypeptides, one of 50 kd (p50) which is involved in DNA binding, and another of 65 kd (p65) which is involved in interaction with IκB, a cytosolic inhibitor of NFκB (24; 25). The recent cloning of the gene encoding p50 has identified it as a member of the rel family of genes (26; 27; 22). Protein sequence analysis also indicates that p65 may be a member of the rel family of proteins. p50 has been speculated to form heterodimers with other members of the rel family of proteins and in this way transactivate different sets of genes (27). Cloning of the genes encoding the p65 and IκB portion of the NFκB complex are likely to follow. The examination of the interactions between these proteins and other members of the rel family of proteins should greatly increase our understanding of how NFκB functions. Of particular interest is the involvement of NFκB in cytokine-induced expression of several genes (19;20). The results of our study demonstrate the importance of an NFκB-like factor in cytokine-induced expression of the ELAM-1 gene. An understanding of the interactions between NFκB and other members of the rel family of proteins should assist in further determining the role of the NFκB-like factor in control of ELAM-1 gene transcription.

This study, which identifies cis-acting elements involved in cytokine induction of ELAM-1 gene expression, represents the first examination of the promoter/enhancer elements of one of the expanding family of endothelial cell adhesion molecules. Given the similarity between several members in their cytokine-induced protein expression, the implication of NFκB-like factors in cytokine induction of other members of this family of proteins is possible.

Interestingly, two regions were essential for cytokine-induced expression of the ELAM-1 gene. Therefore, induction of the ELAM-1 gene by cytokines appears to be achieved through cooperative interactions between at least these two distinct elements. Similar observations have been made in other genes whose transcription is controlled by NFκB. In the case of the b-interferon gene, viral induction involves induced binding of two factors, one being NFκB, to two distinct elements (PRDI and PRDII) within the promoter of the gene. The two factors/elements act together to impart viral inducibility upon the gene. A single copy of either element alone is insufficient for viral induction (28 and 29). Similarly, activation of the IL-2 receptor gene is controlled by NFκB and a second element downstream of the NFκB binding site (30). Activation of the ELAM-1 gene by an NFκB-like factor appears likely to depend upon other trans-activator(s).

ELAM-1 appears only to be expressed on cytokine-induced endothelial cells (2). This is supported by our observation that the ELAM-1 gene is not expressed in either uninduced or cytokine-induced human epithelial cells (HeLa cells). However, we found the pattern of IL-1-induced expression of our ELAM-1/reporter gene chimeras in HeLa cells was very similar to that observed in endothelial cells. No element between nucleotides −741 to +80 of the gene was found to be sufficient to direct cell specific expression of ELAM-1. We conclude that the element(s) involved in conferring cell specific expression are likely to be found elsewhere in the gene. Cell specificity is likely to be conferred by a repressor of cytokine-induced expression in non-endothelial cells. This mechanism of gene regulation has been observed for the Ig κ light chain enhancer. The NFκB site from the κ light chain enhancer alone stimulates transcription in a non-tissue specific manner. However, the entire κ enhancer is inducible only in B lymphocytes. This tissue specificity is due to a repressor sequence within the enhancer, upstream of the NFκB binding site, which inhibits the NFκB-induced expression in non-B cells (17). We are currently examining this possibility for ELAM-1 using additional upstream sequences in an attempt to identify an element conferring tissue specificity.

ACKNOWLEDGEMENTS

We thank Dr. Alain Israel for kindly providing the antiserum against the p50 subunit of NFκB and Biogen for providing the plasmid containing von Willebrand factor cDNA. We also thank Anne-Marie Schenk, Dominique Ayer, Elisabeth Allet, Sandra Stinnett and Steve Haneline for their excellent technical assistance.

REFERENCES

1. Osborn, L. (1990) Cell, 62, 3−6.
2. Bevilacqua, M.P., Pober, J.S., Mendrick, D.L., Cotran, R.S., Gimbrone, M.A. Jr. (1987) Proc. Natl. Acad. Sci. USA, 84, 9238−9242.
3. Bevilacqua, M.P., Stengelin, S., Gimbrone, M.A., Jr., Seed, B. (1989) Science, 243, 1160−1165.
4. Springer, T.A. (1990) Nature, 346, 425−434.
5. Mitchell, P.J. and Tjian, R. (1989) Science, 245, 371−378.
6. Dynan, W.S. (1989) Cell, 58, 1−4.
7. Berk, A.J. and Schmidt, M.C. (1990) Genes & Development, 4, 151−155.
8. Shaw, P.E. (1990) The New Biologist, 2, 111−118.
9. Greenberg, M.E. and Ziff, E.B. (1984) Nature, 311, 433−438.
10. Chirgwin, J.M., Pryzabyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry, 18, 5294−5299.

11. Ansubel, F.M., Brent, R., Kingston, R.E., Moore, D.D. and Seidman, J.G. (1989) Current Protocols in Molecular Biology (Greene Publishing Assoc. and Wiley Interscience, New York).
12. Cullen, B.R. (1987) Methods in Enzymology, 152, 692–693.
13. Whelan, J., Poon, D., Weil, P.A. and Stein, R. (1989) Mol. Cell. Biol., 9, 3253–5259.
14. Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell. Biol., 2, 1044–1051.
15. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nuc. Acids Res., 11, 1475–1489.
16. Dorn, A., Bollekens, J., Staub, A., Benoist, C. and Mathis, D. (1987) Cell, 50, 863–872.
17. Lenardo, M.J. and Baltimore, D. (1989) Cell, 58, 227–229.
18. Breathnach, R. and Chambon, P. (1981) Ann. Rev. Biochem., 50, 349–383.
19. Edbrooke, M.R., Burt, D.W., Cheshire, J.K. and Woo, P. (1989) Mol. Cell. Biol., 9, 1908–1916.
20. Osborn, L., Kunkel, S. and Nabel, G.J. (1989) Proc. Natl. Acad. Sci. USA, 86, 2336–2340.
21. Hooft van Huijsduijnen, R., Li, X.Y., Black, D., Matthes, H., Benoist, C. and Mathis, D. (1990) EMBO J., 9, 3119–3127.
22. Kieran, M., Blank, V., Logeat, F., Vandekerchkhove, J., Lottspeich, F., Le Bail, O., Urban, M.B., Kourilsky, P., Baeuerle, P.A. and Israël, A. (1990) Cell, 62, 1007–1018.
23. Dixit, V.M., Green, S., Sarma, V., Holzman, L.B., Wolf, F.W., O'Rourke, K., Ward, P.A., Prochownik, E.V. and Marks, R.M. (1990) J. Biol. Chem., 265, 2973–2978.
24. Baeuerle, P.A. and Baltimore, D. (1988a) Science, 242, 540–546.
25. Baeuerle, P.A. and Baltimore, D. (1988b) Cell, 53, 211–217.
26. Bours, V., Villalobos, J., Burd, P.R., Kelly, K., Siebenlist, N. (1990) Nature, 348, 76–80.
27. Ghosh, S., Gifford, A.M., Riviere, L.R., Tempst, P., Nolan, G.P. and Baltimore, D. (1990) Cell, 62, 1019–1029.
28. Fan, C.M. and Maniatis, T. (1989) EMBO J., 8, 101–110.
29. Lenardo, M.J., Fan, C.M., Maniatis, T. and Baltimore, D. (1989) Cell, 57, 287–294.
30. Cross, S.L., Halden, N.F., Lenardo, M.J., Leonard, W.J. (1989) Science, 244, 466–469.