MOLECULAR AND CELLULAR BIOLOGY, Sept. 1994, p. 5701–5709
0270-7306/94/$04.00+0
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Vol. 14, No. 9

Cooperativity between Two NF-κB Complexes, Mediated by High-Mobility-Group Protein I(Y), Is Essential for Cytokine-Induced Expression of the E-Selectin Promoter

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Received 20 April 1994/Returned for modification 10 May 1994/Accepted 26 May 1994

Cytokine-induced expression of the E-selectin gene requires the promoter binding and interaction of the transcription factors NF-κB and ATF. Here we have further analyzed the E-selectin promoter and revealed an additional region (nucleotides −140 to −105 [−140/−105]) which is essential in controlling promoter activation by cytokines. We identified high-mobility-group protein I(Y) [HMG-I(Y)] interacting specifically at two sites within this region. We noted that one of the HMG-I(Y)-binding sites overlaps a sequence element (−127/−118) diverging at only one position from the NF-κB consensus binding sequence. This led us to ask whether the −127/−118 element represents a second functional NF-κB-binding site within the E-selectin promoter. Using specific antisera, we show that p50, p65, and, interestingly, RelB are components of the complex interacting at this site. Mutational analysis of the −127/−118 NF-κB site indicates that both NF-κB and HMG-I(Y) binding at this site are essential for interleukin-1 induction of the promoter. We demonstrate that the binding affinity of the p50 subunit of NF-κB to both NF-κB sites within the E-selectin promoter is significantly enhanced by HMG-I(Y). In addition, an essential role for cooperative interaction between the two NF-κB complexes is shown by the requirement for both NF-κB sites to mediate E-selectin promoter activation by interleukin-1 and p50/p65 expression. We conclude that HMG-I(Y) mediates binding of a distinct NF-κB complex at two sites within the E-selectin promoter. Furthermore, a unique cooperativity between these NF-κB complexes is essential for induced E-selectin expression. These results suggest mechanisms by which NF-κB complexes are involved in specific gene activation.

When activated under physiological or pathological conditions, leukocytes transiently adhere to the vascular endothelium. This is an important step in leukocyte migration from the vascular compartment to extravascular tissues. Leukocyte adhesion is thus critical in the development of immune and inflammatory responses. This process requires the expression of adhesion proteins on the endothelial cell surface (for reviews, see references 7 and 46). E-selectin, a member of the selectin family of cell surface glycoproteins, is one of the important endothelial cell adhesion proteins (for a review, see reference 29). The E-selectin protein is rapidly and transiently expressed on the endothelial cell surface following cytokine induction and plays a central role in the extravasation of both neutrophils and a subset of T lymphocytes (5, 38). Transcription of the gene encoding E-selectin is tightly regulated in a cell-specific manner, being expressed exclusively on endothelial cells following exposure to either interleukin-1β (IL-1β) or tumor necrosis factor alpha (5, 17, 51).

Our work has focused on understanding the mechanisms regulating induced E-selectin expression at the level of gene transcription. In particular, we have focused on identifying the transcription factors involved and have examined how specific interactions between these factors may bring about specific regulation of the gene. Previously, we and others have shown that IL-1β and tumor necrosis factor alpha induce the binding of NF-κB to a site at nucleotides −94 to −85 (−94/−85) upstream of the E-selectin transcription start site. Binding of NF-κB to this position is essential in mediating cytokine induction of the gene (8, 34, 51).

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The two subunits of the originally defined NF-κB complex (p50 [NF-κB1] and p65 [RelA] subunits) are members of the Rel family of transcription factors. These proteins have extensive amino acid homology with the N-terminal 300 amino acids of the v-Rel oncoprotein and its cellular homolog, c-Rel (for reviews, see references 6, 18, and 21). Other members of this family include NF-κB2 and RelB (43, 44). Members of the Rel family can both homodimerize and heterodimerize (for a review, see reference 32). However, there is a selectivity in their ability to heterodimerize, since RelB is unable to heterodimerize with p65 (41). Furthermore, the p50/RelB heterodimer has an activity distinct from that of the p50/p65 heterodimer in primary lymphoid cells (31). Thus, different combinations of the Rel family of transcription factors may be involved in specific cellular transcriptional responses (32).

NF-κB is constitutively active in the nucleus of B lymphocytes and possibly some T-cell lines and mature macrophages (for a review, see reference 21). However, in most cell types NF-κB is retained in the cytoplasm in a complex with I-κB and is activated in response to a variety of inducing agents (e.g., cytokines, viruses, and stress factors). The active factor then plays a central role in the rapid induction of a wide variety of genes, particularly those involved in immune and inflammatory responses (for reviews, see references 2 and 30).

In light of the fact that NF-κB is involved in the induced expression of a wide variety of genes, a key question has been how NF-κB is involved in the specific activation of these genes. We demonstrated that interaction of NF-κB at the −94/−85 position alone was not sufficient to mediate induction of the E-selectin gene (51). In an increasing number of examples, protein-protein associations between various types of transcriptional regulatory factors appear to determine the specificity of gene activation (12, 26, 47, 48). In addition to interaction

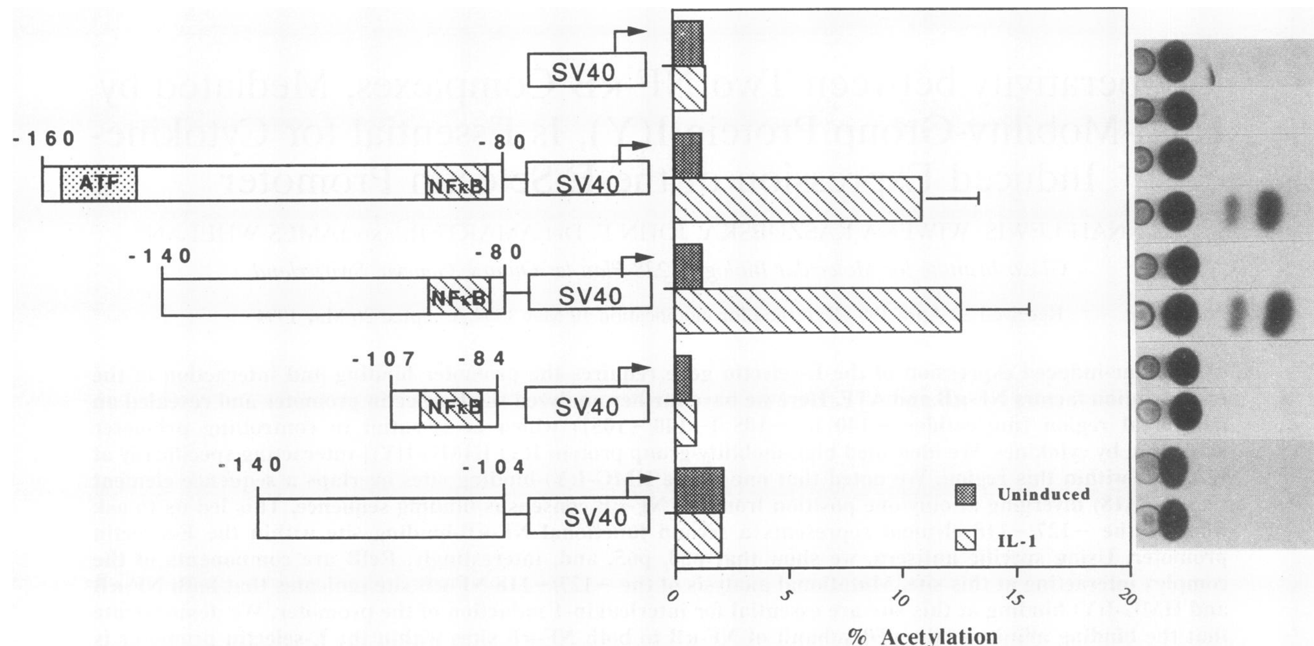

FIG. 1. The control element(s) required for IL-1 induction is contained within the −140/−104 region of the E-selectin promoter. The CAT enzymatic activities of constructs containing various E-selectin promoter fragments, upstream of a minimal SV40 promoter were assayed following transient transfection in endothelial cells (HUVEC). The cells were treated with IL-1 (20 U/ml) 24 h prior to extract preparation. Results are the means ± standard errors of the mean for at least three independent experiments. Results of a representative experiment are shown.

with NF-κB, induction of the E-selectin promoter also involves interaction of an ATF complex at a position upstream of the NF-κB site (−154/−147). Furthermore, protein-protein interaction between these promoter-bound complexes is important in mediating cytokine activation (23, 25, 37).

The results of the present study demonstrate a further level of complexity in specific activation of the E-selectin gene by NF-κB. Here we define a second NF-κB-binding site within the E-selectin promoter. Furthermore, RelB appears to be a component of the E-selectin promoter binding NF-κB complexes. High-mobility-group protein I(Y) [HMG-I(Y)] plays a central role in E-selectin promoter activation by mediating NF-κB binding. We demonstrate that cooperativity between the two NF-κB complexes is essential in mediating IL-1 induction of the E-selectin gene. These results demonstrate a distinct role for NF-κB in activation of E-selectin expression.

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 MCDB 131 medium supplemented with epidermal growth factor (10 ng/ml), hydrocortisone (1 ng/ml), 2% fetal bovine serum, and 0.4% bovine brain extract (Clonetics, Calif.).

Plasmid constructs. The E-selectin–minimal simian virus 40 (SV40) promoter–chloramphenicol acetyltransferase (CAT) reporter vectors were made by subcloning the promoter fragments indicated below into the *Bgl*II restriction site of the plasmid pCAT-Promoter (Promega). The vectors with mutations within the NF-κB II site were generated by oligonucleotide site-directed mutagenesis as described by Clontech. The mutation of the NF-κB I site has been described previously (51). The p50 eukaryotic expression plasmid was made by subcloning the *Eco*RI-*Xba*I partial cDNA fragment of p105 (27) from the Bluescript vector into an expression plasmid, pSG5, containing the SV40 promoter (20). This expression plasmid encodes the N-terminal 502 amino acids of p50. The cytomegalovirus promoter containing eukaryotic expression plasmid soCMIN was used for expression of full-length p65 (42).

Expression and purification of recombinant proteins. For preparation of glutathione *S*-transferase–HMG-I (GST–HMG-I), the HMG-I cDNA was subcloned into the bacterial expression vector pGEX-B (50). Proteins were purified as described by Smith and Johnson (45) with the following modifications. An overnight culture of *Escherichia coli* (DH5α) transformed with the recombinant pGEX plasmid was diluted 1:50 in 500 ml of Luria broth and grown at 37°C until the optical density at 600 nm reached 0.5. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added to a final concentration of 0.5 mM, and the culture was incubated at 37°C for an additional 4 h. Cells were pelleted and resuspended in 20 ml of PBSE (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 1 mM EDTA). Cells were lysed by two passages through a French pressure cell, and Triton X-100 was added to 1%; this was followed by centrifugation at 10,000 × *g* for 30 min at 4°C. The supernatant was mixed with 0.5 ml of 50% glutathione agarose beads for 30 min at 4°C. The beads were washed five times with 15 ml of PBSE. GST–HMG-I was eluted with free glutathione by using two 250-μl aliquots of elution buffer (50 mM Tris-HCl [pH 8], 500 mM NaCl, 10 mM reduced glutathione) after 30-min incubations.

HMG-I cDNA was also cloned into the pET15b vector and transformed into *E. coli* BL21(DE3). The histidine-tagged HMG-I was purified by using a nickel-chelating column (Qiagen) as previously described (50).

Nuclear extract preparation and band shift analysis. Nuclear extracts were prepared as described previously (10) with modifications as described elsewhere (11). Binding reaction

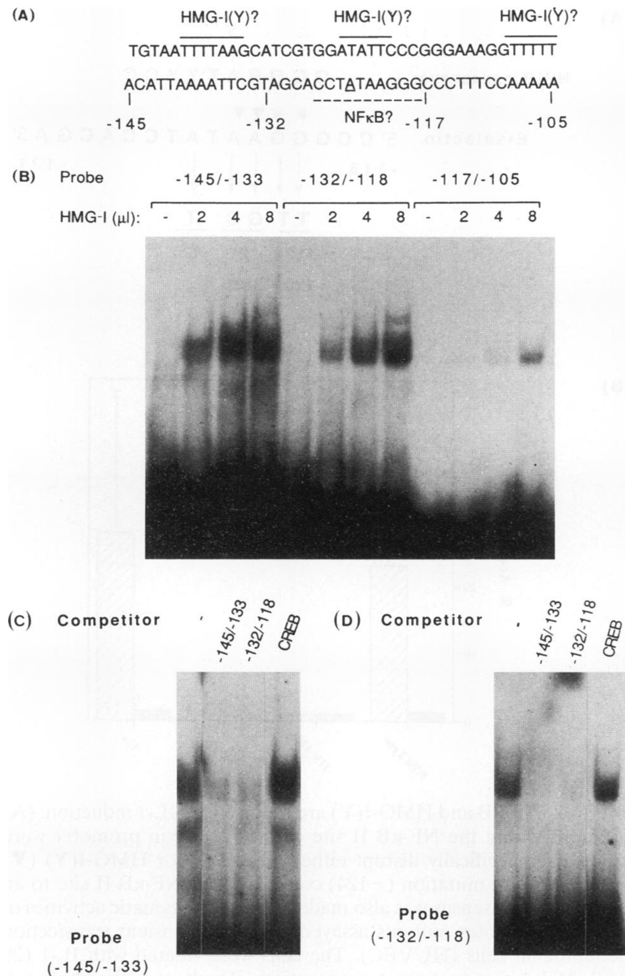

FIG. 2. Specific binding of GST–HMG-I to E-selectin promoter. (A) Potential binding sites for HMG-I(Y) and NF-κB within the −140/−105 region. (B) Increasing amounts of bacterially expressed and purified GST–HMG-I were used in band shift analysis. End-labeled oligomers corresponding to the indicated nucleotides within the E-selectin promoter were used as probes. (C and D) Specificity of purified GST–HMG-I binding to the two most 5′ oligomers assessed by competition with unlabeled oligomers (75-fold molar excess) corresponding to the sites 145/−133 and −132/−118 or with an unrelated oligomer (CREB).

mixtures with nuclear extracts and GST–HMG-I contained 50 mM NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5), 0.1 μg of poly(dG-dC) per μl, 10% (vol/vol) glycerol, and approximately 1 to 5 μg of protein. Binding reaction mixtures with purified p50 and histidine-tagged HMG-I contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, and 1 mg of bovine serum albumin per ml. Each 20-μl reaction mixture was incubated on ice for 20 to 30 min following addition of approximately 100,000 cpm of ^{32}P-labeled oligonucleotide probe. Competition experiments were carried out by preincubating the reaction mixture with excess unlabeled oligonucleotides for 10 min on ice prior to addition of labeled probe. Antibodies (Santa Cruz Biotechnology, Inc.) were preincubated with extracts for 30 min on ice. When used, HMG-I and p50 were preincubated for 30 min on ice prior to addition of labeled probe. Samples were then subjected to electrophoretic separation on a 4% nondenaturing polyacrylamide gel. The gels were dried and subjected to autoradiography.

DNA transfection and CAT analysis. Approximately 12 h prior to transfection, HUVEC were plated at a density of 10^6 cells per 60-mm-diameter plate. Transfection of plasmid DNA into HUVEC was carried out by the DEAE-dextran method as described previously (9). CAT assays were carried out 48 h posttransfection essentially as described previously (19). CAT enzymatic activity was quantitated by measuring the conversion of chloramphenicol to its acetylated forms by using an Ambis radioanalytic imaging system according to the manufacturer's instructions.

RESULTS

An additional control region is essential for cytokine-induced E-selectin expression. To further understand the mechanisms by which members of the ATF and Rel families of transcription factors specifically control E-selectin gene expression, we subcloned a fragment spanning the binding sites for these factors (nucleotides −160 to −80) upstream of the SV40 minimal promoter. This region of the E-selectin promoter was sufficient to impart cytokine inducibility upon the heterologous promoter, with the level of promoter activity being increased approximately ninefold following IL-1 induction (Fig. 1, −160/−80). This level of induction is similar to that previously observed with the wild-type E-selectin promoter (25). This result suggests that all the elements required for cytokine induction are contained within this region of the promoter.

Surprisingly, deletion of the ATF-binding site (position −154/−147) did not result in any decrease in IL-1 inducibility in the context of a heterologous promoter (Fig. 1, −140/−80). Previously, we have shown that mutation of the ATF-binding site in the context of the wild-type E-selectin promoter results in almost complete loss of IL-1 inducibility (23). This result suggests that when this region of the E-selectin promoter is placed upstream of a minimal heterologous promoter, the specific requirement for the ATF complex in imparting IL-1 induction is lost. Published results suggest that the basal transcription initiation complex (TFIID complex) formed on the TATA boxes of different promoters is a heterogeneous multisubunit complex and that different initiation complexes require distinct upstream factors for specific gene activity (15, 35).

When the fragment upstream of the heterologous promoter was deleted, so as to span only the previously defined NF-κB (−94/−85) site, no IL-1 induction was observed (Fig. 1, −107/−84). This led us to conclude that the region immediately upstream of this NF-κB site contains an element which is essential for IL-1 induction of the heterologous promoter. However, placement of a fragment spanning this region (−140/−104) upstream of the heterologous promoter did not impart IL-1 inducibility upon the promoter. We conclude that the proteins binding these elements (NF-κB [−94/−85] and −140/−104) alone are unable to confer IL-1 induction. Rather, the protein(s) binding to the −140/−104 region cooperates with NF-κB to bring about induction.

Identification of HMG-I(Y) as one of the proteins interacting within the −140/−104 region. Band shift analysis was performed to examine whether specific protein complexes bound to the −140/−104 region of the E-selectin promoter. With the −140/−104 region as a probe, a specific yet slightly smeared band shift pattern was observed (data not shown). This pattern in the band shift suggested that more than one

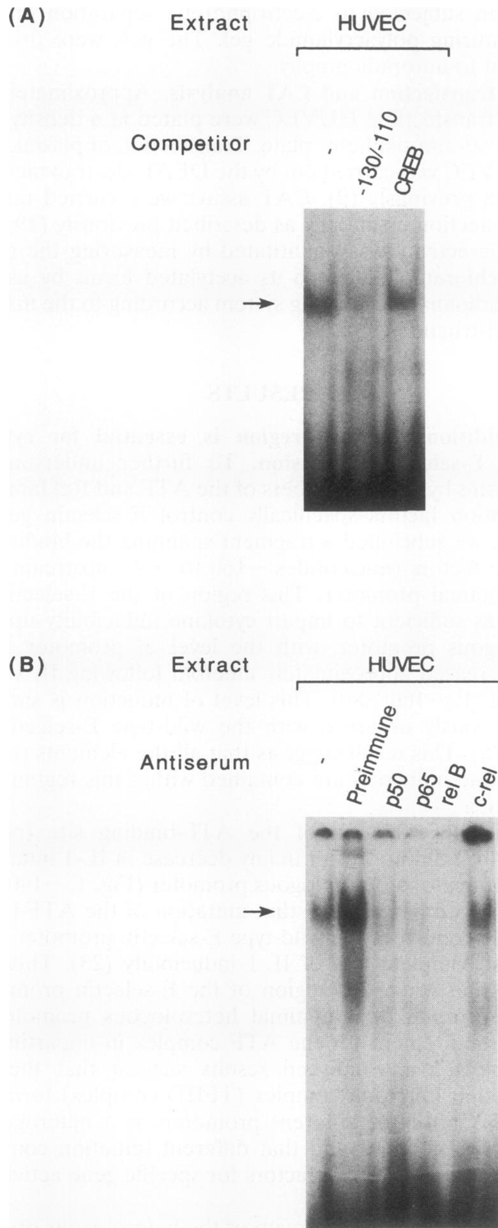

FIG. 3. Members of the Rel family of factors bind to the −130/−110 region of the E-selectin promoter. (A) Band shift analysis with nuclear extracts prepared from IL-1-induced HUVEC and a labeled oligomer corresponding to nucleotides −130 to −110. Specificity of binding was assessed by competition with unlabeled −130/−110 oligomer and an unrelated oligomer (CREB) (75-fold molar excess). (B) Band shift analysis with nuclear extracts prepared from IL-1-induced HUVEC to examine the effect of preimmune serum or antisera raised against members of the Rel family of factors. Arrows, positions of the complex.

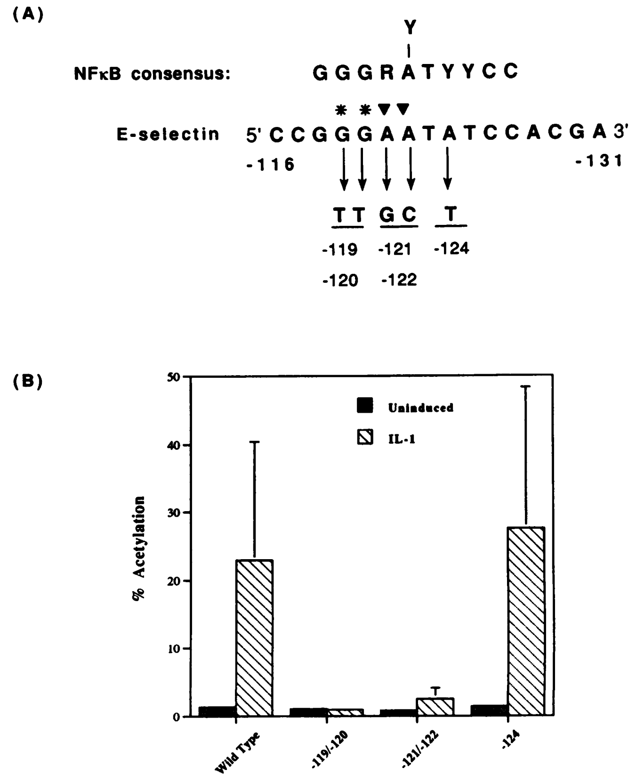

FIG. 4. NF-κB and HMG-I(Y) are required for IL-1 induction. (A) Mutations within the NF-κB II site of the E-selectin promoter were designed to specifically disrupt either NF-κB (*) or HMG-I(Y) (▼) binding. A point mutation (−124) converting the NF-κB II site to an exact NF-κB consensus was also made. (B) CAT enzymatic activities of E-selectin promoter mutants assayed following transient transfection in endothelial cells (HUVEC). The cells were treated with IL-1 (20 U/ml) 24 h prior to extract preparation. Results are the means ± standard errors of the mean for at least three independent experiments. There is a statistically significant difference in the activity of the wild type compared with those of the −119/−120 and −121/−122 mutants ($P < 0.003$).

protein complex is interacting with this region of the promoter. In an attempt to identify these binding proteins, an endothelial λgt11 expression library was screened with a labeled fragment covering this region of the promoter. A positive clone encoding HMG-Y was identified (data not shown). HMG-I and an isoform, HMG-I(Y), are DNA-binding proteins that preferentially bind to the minor groove of certain A-T-rich sequence elements (13, 24, 39). Three potential HMG-I(Y)-binding sites are present within the −140/−104 region (Fig. 2A). To examine the ability of HMG-I(Y) to bind to these sites, we used purified GST–HMG-I(Y) in a band shift analysis with three labeled probes, each of which spanned one of the potential HMG-I(Y) sites. With increasing amounts of purified protein, it can be seen that HMG-I(Y) binds strongly to the two most 5′ sites (−140/−135 and −125/−121) and binds significantly less efficiently to the most 3′ site (−109/−105) (Fig. 2B). The specificity of HMG-I(Y) binding to the two most 5′ sites is demonstrated by the competition for HMG-I(Y) binding by unlabeled −145/−133 and −132/−118 competitors but not by the nonspecific competitor (CREB) (Fig. 2C and D). We conclude that HMG-I(Y) binds specifically to the E-selectin promoter on at least two sites.

A Rel protein complex interacts with an element overlapping one of the HMG-I(Y)-binding sites. We noted that the central HMG-I(Y) site (−125/−121) within the −140/−104 region overlaps a sequence element, on the noncoding strand, which diverges from the NF-κB consensus sequence at only one position (−124) (Fig. 2A). Furthermore, band shift analysis with nuclear extracts prepared from IL-1-induced HUVEC

and a labeled probe spanning this site (−130/−110) demonstrated specific binding of a slowly migrating complex to this element (Fig. 3A). The specificity of interaction of this complex is illustrated by the ability of the unlabeled −130/−110 fragment but not a nonspecific fragment (CREB) to compete for the complex. HMG-I(Y) is a low-molecular-weight protein (predicted molecular weight, approximately 10,000) which migrates rapidly on a nondenaturing gel (50). Our observation of a slowly migrating complex binding the −130/−110 probe suggested that proteins, in addition to HMG-I(Y), may be components of this complex. Using antibodies raised against Rel proteins, we investigated whether members of the Rel family of factors are components of this complex. Formation of the complex was unaffected by preincubation of the HUVEC nuclear extract with preimmune serum. However, complex formation was inhibited by preincubation of the extracts with antibodies raised against p50, p65, and RelB (Fig. 3B). The complex was unaffected by c-Rel antiserum, indicating that c-Rel is not a component of this complex. This result confirms that the protein complex interacting at this site (hereafter referred to as the NF-κB II site) within the E-selectin promoter is composed, at least in part, of Rel proteins.

The presence of RelB in the complex is an interesting observation and suggests that the Rel complex interacting at this site contains more than a p50/p65 heterodimer. It is not clear whether the complex is a mix of p50/p65 and p50/RelB heterodimers or whether it represents a higher-order complex. Using specific RelB antiserum, we have also demonstrated that RelB is a component of the NF-κB complex interacting at the −94/−85 site (hereafter referred to as the NF-κB I site) (data not shown). The potential role of RelB in control of E-selectin expression is currently being investigated.

Both Rel and HMG-I(Y) proteins interacting at the NF-κB II site are essential for promoter activation. To further examine the roles of Rel and HMG-I(Y) proteins interacting at the NF-κB II site, we undertook a detailed mutational analysis of the site. We constructed two mutations to specifically disrupt either NF-κB binding or HMG-I(Y) binding. In addition, we made a point mutation to convert the NF-κB II site to a true NF-κB consensus sequence (Fig. 4A). The effects of these mutations on activation of the E-selectin promoter by IL-1 were then examined.

Transversion mutations of two conserved G nucleotides at the 5′ end of the NF-κB consensus sequence (positions −119 and −120), which disrupt NF-κB binding without affecting HMG-I(Y) binding (Fig. 5; compare lanes 1 and 2 with lanes 7 and 8), resulted in complete inhibition of IL-1 induction of the E-selectin promoter (Fig. 4B, −119/−120). This result demonstrates the importance of NF-κB interaction at this site in control of cytokine induction of the promoter. To address the importance of HMG-I(Y) interaction at this position within the promoter, we mutated the two A nucleotides at positions −121 and −122 to G and C nucleotides, respectively. This mutation disrupts the A/T core required for HMG-I(Y) binding while maintaining the NF-κB consensus (Fig. 4A), thereby disrupting HMG-I(Y) binding without affecting the binding of NF-κB at this site (data not shown). This mutation, although not completely blocking IL-1 induction, results in a 10-fold decrease in activation (Fig. 4B, −121/−122). We conclude that the HMG-I(Y) interaction at the NF-κB II site plays an important role in controlling cytokine induction of the promoter.

The NF-κB II site diverges at a single position from the defined NF-κB consensus sequence, having an adenine instead of a pyrimidine at position −124. We hypothesized that if this divergent nucleotide was sufficient to significantly reduce NF-κB binding at this site, a mutation to convert this site to a true consensus sequence should result in enhanced NF-κB binding and subsequently increase IL-1 induction of the promoter. The promoter with such a point mutation (−124) was induced by IL-1 to a level equivalent to that observed with the wild-type E-selectin promoter (Fig. 4B). We conclude that the wild-type promoter, with a single nucleotide diverging from the consensus sequence in the NF-κB II site, binds both NF-κB and HMG-I(Y). Moreover, both of these factors are essential for IL-1 induction. A functional cooperativity between NF-κB and HMG-I(Y) is required in activation of the E-selectin promoter.

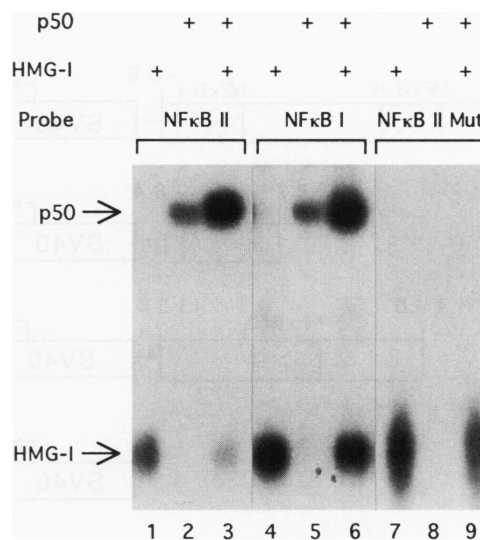

FIG. 5. HMG-I(Y) enhances p50 binding to E-selectin NF-κB sites. Band shift analysis was carried out with bacterially expressed and purified p50 and histidine-tagged HMG-I(Y). Labeled wild-type or mutant oligomers spanning one of the two E-selectin NF-κB sites were used as probes. The wild-type NF-κB II probe spans nucleotides −130/−110 of the E-selectin promoter. The NF-κB II Mut probe carries G-to-T substitutions at positions −119 and −120 (see Fig. 4A). The wild-type NF-κB I probe spans nucleotides −104 to −75 of the E-selectin promoter.

HMG-I(Y) mediates NF-κB binding to the E-selectin promoter. We determined the ability of Rel proteins to bind the E-selectin NF-κB II site and the role of HMG-I(Y), using band shift analysis and recombinant purified p50 and HMG-I(Y). As shown above (Fig. 2B), HMG-I(Y) alone binds to the NF-κB II probe (Fig. 5, lane 1). Interestingly, HMG-I(Y) also interacts with a probe spanning the NF-κB I site (Fig. 5, lane 4). As predicted, p50 alone binds to both NF-κB sites (Fig. 5, lanes 2 and 5). When recombinant p50 and HMG-I(Y) are mixed prior to incubation with the labeled probe, a significant increase in the binding of p50 to both sites is observed (Fig. 5, lanes 3 and 6). As a control, mutation of two G nucleotides (−119/−120) at the 5′ end of the NF-κB II consensus sequence blocks p50 binding without affecting HMG-I(Y) binding (Fig. 5, lanes 7 and 8). Moreover, HMG-I(Y) is unable to facilitate p50 binding to this mutated probe. We conclude that HMG-I(Y) binds the two NF-κB sites within the E-selectin promoter and plays an important role in E-selectin induction by mediating enhanced NF-κB binding to both sites. Thus, the role of HMG-I(Y) appears to be similar to that previously observed with the beta interferon (IFN-β) gene (50).

Cooperativity between NF-κB complexes is essential in

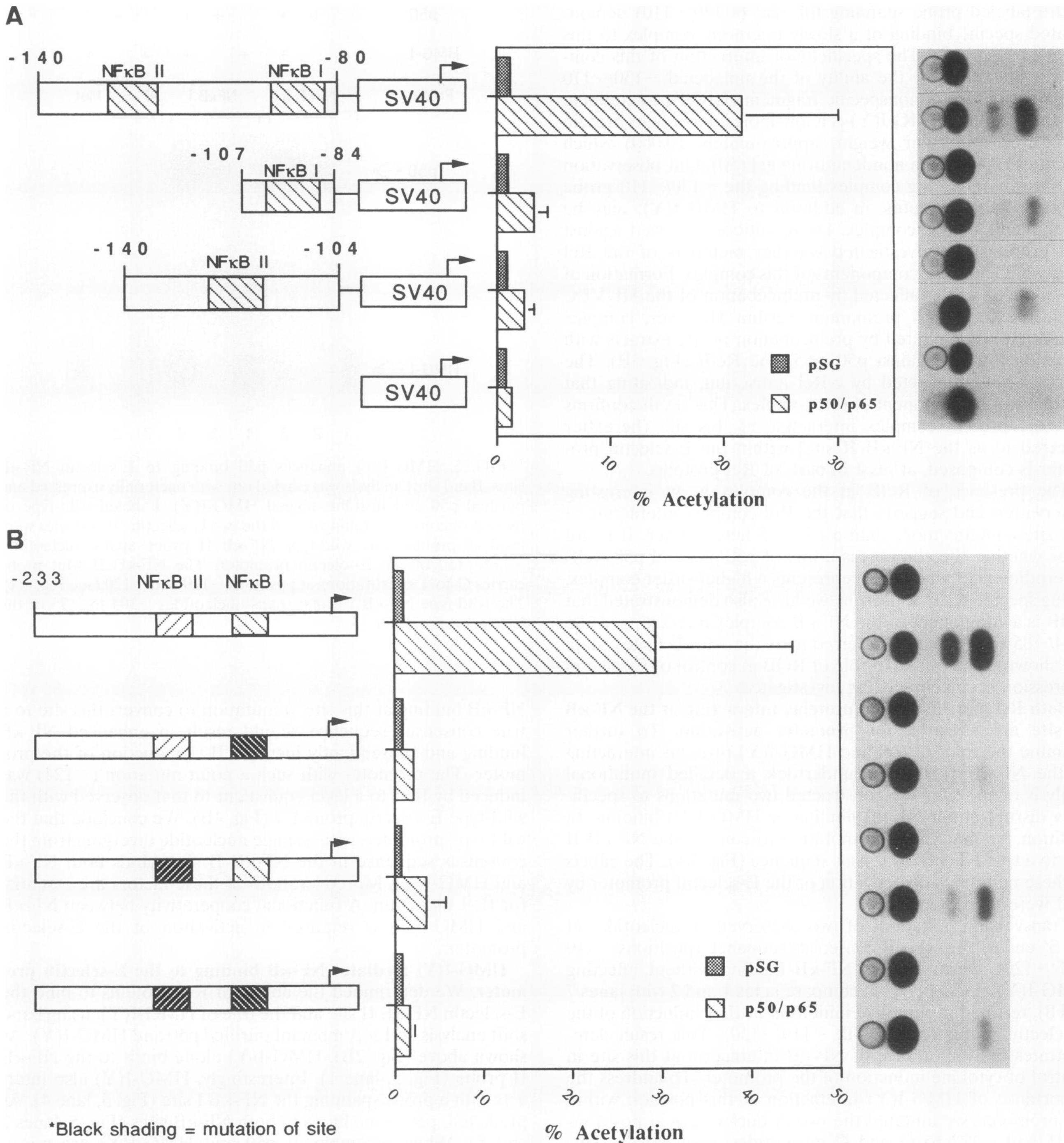

FIG. 6. Both NF-κB I and II sites are required to mediate activation by p50/p65. HUVEC were transfected with the indicated E-selectin promoter–CAT reporter plasmids (5 μg) in combination with either pSG5 expression plasmid alone (4 μg) or expression plasmids encoding the two subunits of NF-κB (2 μg of each). Promoter activity was determined by CAT analysis. Results are the means ± standard errors of the mean for at least three independent experiments. Results of a representative experiment are shown.

mediating E-selectin promoter activation. Above, we demonstrated that fragments spanning either of the NF-κB sites alone, placed upstream of a minimal heterologous promoter, are unable to confer IL-1 inducibility (Fig. 1). However, when combined, the two NF-κB sites conferred strong IL-1 induction upon the minimal promoter. This observation strongly suggests that cooperative interaction between these NF-κB complexes is essential for IL-1 induction of the promoter.

To further address this cooperative interaction between the NF-κB complexes, we asked whether expressed p50/p65 can activate various E-selectin–heterologous promoter constructs. In agreement with the results obtained following IL-1 induc-

tion, we observed strong activation (approximately 20-fold) of the construct containing both NF-κB sites (I and II) upstream of a minimal heterologous promoter. However, constructs containing either NF-κB site alone were induced to only very low levels (approximately two- to threefold) by p50/p65 (Fig. 6A). p50/p65 expression fails to activate the minimal promoter. These results support the hypothesis that induction of the E-selectin–heterologous promoter constructs following IL-1 treatment of HUVEC involves a cooperative interaction between the two NF-κB complexes interacting on the promoter.

In addition, we wanted to examine this cooperativity in the context of the wild-type E-selectin promoter. Therefore, we determined the ability of p50/p65 to activate E-selectin promoter constructs having mutations in the two NF-κB sites, either individually or in combination. The wild-type E-selectin promoter is strongly activated (approximately 26-fold) by p50/p65 expression (Fig. 6B), in agreement with our previous report (25). Mutation of the NF-κB I site resulted in almost complete inhibition of p50/p65 induction (less than twofold induction). Likewise, mutation of the NF-κB II site also resulted in a very significant reduction in p50/p65 activation (less than fivefold induction). As expected, the promoter construct containing mutations in both NF-κB sites is not induced by p50/p65. These results demonstrate that both NF-κB sites within the E-selectin promoter are required to mediate activation by p50/p65. Furthermore, cooperativity between the two NF-κB complexes is essential for promoter activation.

DISCUSSION

Cooperativity between different families of transcription factors has recently been recognized as an important element in regulating gene expression. Cooperative interactions between NF-κB and SP-1 (36), NF-IL6 (4), Fos/Jun (48), and C/EBP (49) factors have recently been demonstrated. We have previously shown that interactions between NF-κB and members of the ATF/CRE family play a critical role in the induced expression of E-selectin (25). Here we demonstrate functional cooperativity between two NF-κB complexes which is essential for IL-1 induction of the E-selectin promoter. A role for multiple NF-κB sites has been observed in the regulation of several other genes. The enhancer of the long terminal repeat of human immunodeficiency virus type 1 contains two NF-κB sites, both of which contribute to the transcriptional activity (16). Three NF-κB complexes play a role in the constitutive murine c-Rel expression in B cells (22). However, unlike the E-selectin gene, where mutation of either NF-κB site completely blocks induction, mutation of individual sites within these other genes reduces rather than blocks expression. Therefore, to date E-selectin represents a unique example of a promoter which has an absolute requirement for more than one NF-κB complex in promoter activation.

We found that both NF-κB sites within the E-selectin promoter overlap HMG-I(Y)-binding sites. We show that HMG-I(Y) is involved in increasing the binding of p50 to these sites. The involvement of HMG-I(Y) in establishing synergy between transcription factors by enhancing their binding to DNA has also been reported for the human IFN-β promoter (12, 50). The viral inducibility of the IFN-β promoter is dependent on the interactions of HMG-I(Y) with NF-κB and ATF2. However, HMG-I(Y) does not function as a general cofactor, since it is not required for NF-κB binding to all κB sites. HMG-I(Y) has been shown not to bind or influence the binding of NF-κB to the immunoglobulin-κB-binding site (50). Therefore, HMG-I(Y) plays an essential role in mediating NF-κB binding and activity at specific NF-κB sites.

Interestingly, the factors controlling virus induction of the IFN-β gene include ATFs as well as NF-κB and HMG-I(Y). Thus, a similar set of factors is involved in both induction of E-selectin by IL-1 and induction of IFN-β by virus. However, the activation of these genes is specific, since the IFN-β promoter is not induced by cytokines and E-selectin is only very weakly induced by virus (49a). The question arises as to how the activation of these two very distinct genes is controlled by a similar set of transcription factors. In addition to the above-mentioned factors, virus induction of the IFN-β gene requires the IRF-1 transcription factor (40). In this study, we have demonstrated that unlike the IFN-β promoter, which has a single NF-κB site, there is an absolute requirement for two NF-κB complexes to activate the E-selectin promoter.

Another level of specificity in activating the E-selectin promoter versus other NF-κB-dependent promoters is likely to be the actual composition of the NF-κB complexes. We report here that the two NF-κB complexes binding the E-selectin promoter contain p50, p65, and RelB. NF-κB complexes containing multiple members of the Rel family of factors have previously been reported. An NF-κB complex has been identified in stimulated T cells; this complex contains four Rel proteins, p50, NF-κB2, p65, and c-Rel (1, 33). Rel proteins may form higher-order complexes, since NF-κB eluted as a complex larger than expected, suggesting tetramer formation, when gel filtration was used (3). The presence of multiple Rel proteins within the complex interacting with the E-selectin promoter may therefore indicate the presence of a tetramer rather than a dimer complex. Alternatively, the complex may be a mix of p50/p65 and p50/RelB heterodimers. These heterodimer complexes may play specific roles in E-selectin expression. In primary lymphoid cells the p50/RelB heterodimer is constitutively active, in contrast to the p50/p65 heterodimer, which is inducible and present in many cell types (31). The presence of RelB in the E-selectin NF-κB complexes provides another potential level of specificity in regulating gene expression.

The NF-κB complex interacting at a particular κB site may be influenced by subtle differences in sequence. Each κB site may preferentially bind a certain set of NF-κB complexes. p50 and p65 homodimers display preferential binding to slightly different DNA sequences (28). As a consequence, the sequence of individual κB sites may play an important role in gene activation by specific NF-κB complexes. The sequence of the NF-κB II site in the E-selectin promoter departs from the consensus sequence at one position. An adenine residue in the seventh position of the consensus sequence is found in the NF-κB II site (Fig. 4A). A pyrimidine is present at this position in 37 of 40 naturally occurring NF-κB sites (for a review, see reference 21). This single nucleotide difference may be sufficient to alter the affinities of certain NF-κB complexes for this site.

Finally, we observed that the ATF complex, although essential for activation of the wild-type E-selectin promoter (23), is not required for IL-1 induction in the context of the minimal SV40 promoter (Fig. 1). Recent reports have suggested that distinct initiation complexes are formed on different TATA box-containing promoters and that each initiation complex requires distinct upstream factors for specific gene activation (15, 35). Recently, c-Rel protein has been shown to associate with the TATA-binding protein of the TFIID complex, indicating that there is direct communication between Rel proteins and the basal initiation complex (26). The importance of interaction of CREB, a member of the leucine zipper family,

with a component of the basal initiation complex has also been demonstrated (14). These results suggest the interesting possibility that the ATF complex may play an essential role in the specific interaction of the NF-κB complexes with the E-selectin promoter initiation complex. However, the ATF complex may be unnecessary in the context of NF-κB interaction with the SV40 initiation complex. This hypothesis is currently under investigation.

The results of this study have allowed us to better understand the complex mechanisms involved in the regulation of E-selectin expression. Specific factors and protein-protein interactions are responsible for achieving a remarkable level of specificity in control of promoter activity. The results we present here also provide further insight into the mechanisms by which NF-κB complexes, through their DNA and protein-protein interactions, can specifically control gene activity.

ACKNOWLEDGMENTS

Hannah Lewis and Wiweka Kaszubska contributed equally to this work.

We thank Dimitris Thanos for providing HMG-I expression vectors and recombinant p50 protein as well as for his helpful suggestions during this work. We thank Ulrike Schindler and Vijay Baichwal (Tularik, Inc.) and Tucker Collins (Brigham and Women's Hospital) for communication of results prior to publication. We also thank Anne-Marie De Raemy-Schenk for her excellent technical assistance and Christopher Herbert for his help in preparation of the figures.

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