Transcription Factor-induced, Phased Bending of the E-selectin Promoter*

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E-selectin is an endothelial adhesion molecule that is critically involved in neutrophil adhesion and recruitment. All DNA elements required for interleukin-1 inducibility have been located in the proximal promoter: an NF-ELAM1/ATF site, two NF-xB sites (I and II), the NF-ELAM2 element and a TATA box. We show here that interleukin-1 induced promoter activity is exquisitely sensitive to the spatial arrangements of these elements. Phasing of the ATF and NF-xB II elements indicates that their relative helix orientation is more important than distance per se. This sensitivity is partly due to a requirement for correctly oriented, transcription factor-induced DNA-bending. (i) Band shift analyses with permuted ATF- and NF-xB elements show that their associated factors all bend DNA. (ii) One can functionally replace the NF-ELAM1/ATF element by a subset of a panel of DNA fragments that contain defined bends in various planes. We conclude that the main role of the factors binding at the NF-ELAM1/ATF element is to alter the conformation of the E-selectin promoter, presumably looping distant enhancer elements into each other’s proximity.

ENDOTHELIAL AND NON-ENDOTHELIAL CELLS (6), WHEREAS TISSUE-SPECIFIC

E-selectin, also known as CD62E or ELAM-1 (1) (for endothelial leukocyte adhesion molecule-1) (1), is a cell surface glycoprotein expressed exclusively on endothelial cells in response to cytokines IL-1β and TNFα (2). E-selectin adheres a subset of leukocytes and is involved in chronic inflammation; aberrant expression of E-selectin is associated with several pathological conditions such as asthma and psoriasis (3), as well as formation of metastases (4). Consistent with its critical role in leukocytes and is involved in chronic inflammation; aberrant expression of E-selectin is associated with several pathological conditions such as asthma and psoriasis (3), as well as formation of metastases (4). Consistent with its critical role in neutrophil adhesion and recruitment, E-selectin expression is transient, peaking at about 4 h after IL-1 induction (5–7). The up and downstream modulation of E-selectin expression occurs principally by altering the gene’s transcription rate, as both the E-selectin protein and mRNA are short-lived (7).

The DNA sequence immediately upstream of the transcribed E-selectin sequence has been intensively studied. It has been shown that only 383 bp upstream promoter sequence is sufficient to drive IL-1 or TNFα-inducible transcription in both endothelial and non-endothelial cells (6), whereas tissue-specific expression is associated with promoter hypo-methylation (8). It has been shown that two NF-xB/HMG I(Y) elements within this proximal E-selectin promoter sequence are essential for IL-1 induction (6, 9). In addition, we have identified two other sequence elements and their binding factors that co-operate with NF-xB for maximal, induced E-selectin transcription (summarized in Fig. 1A). One of these sites, NF-ELAM1/ATF, is bound by members of the bZIP family of transcription factors: ATF-α, ATF-2, ATF-3, c-Jun (10), and CREB (11). Another essential DNA element, NF-ELAM2, is bound by non-identified nuclear proteins (12). These elements and their spacing have been closely conserved among human, mouse and rabbit E-selectin promoters (13, 14). Using a panel of promoter mutants we show here that the spacing of the enhancers is highly critical for promoter activity. This spacing dependence can be explained in two ways. (i) Transcription factors need to bind at the same face of the DNA-helix for optimal protein-protein interaction and co-operation (ii) The bending angle of the promoter induced by transcription factors is changed by 180° when the elements are moved up- or downstream by half-helix turns, resulting in altered promoter activity. The E-selectin promoter is indeed extremely bent by its binding factors; TF-IID (15, 16), various ATF-members (17), HMG I(Y) (18), and NF-xB (19) all have been shown to bend DNA. Furthermore, we show here that the E-selectin promoter is also bent by factors binding the NF-ELAM1/ATF site and a by previously identified p50/p55 form of NF-xB. In order to assess whether this induced E-selectin promoter bending is associated with transcription activity, we replaced the NF-ELAM1/ATF site by a series of intrinsically bent DNA fragments that are not known to bind transcription factors.

EXPERIMENTAL PROCEDURES

Cloning Procedures—The E-selectin promoter mutants in Fig. 1B are derived from 383-CAT (6), in which the CAT gene is driven by 383 bp of the E-selectin promoter. The entire vector was PCR-amplified using the xB- or EL1-oligonucleotides listed in Table I (first 16 on list). Amplification was in 100 µl, total volume, as in (20), using 25 cycles of 1 min at 94 °C, 1.5 min at 50 °C or 60 °C, and 5 min at 72 °C, with 50 ng of template and 2.5 units of Taq polymerase (Perkin-Elmer). The products were phenol-treated, precipitated, purified from agarose gels, digested with either MspI or PstI and ligated into the pBEND2 XbaI-SalI sites, using standard procedures (22).

The vectors with intrinsically bent DNA are derived from p383CAT (23, 24). This vector has 383-bp E-selectin promoter driving expression of the tetracycline-repressor/VP16 fusion protein (25). This vector was PCR-amplified using EL1-mutPCR and N-Ctr1 (see Table I). In the product, the NF-ELAM1/ATF site has been mutated (A to C; G to T, and vice versa), and a unique MluI site was created at its 5′ flank. The curve
Fig. 1. Effect of enhancer spacing on E-selectin promoter activity. Panel A, organization of the E-selectin promoter. The names of the DNA elements (boxed) are indicated above the sequence, the transcription factors known to bind below. Panel B, sequence of promoter constructs in which the NF-ELAM1/ATF (top) or NF-κB II (bottom) element have been moved up- or downstream by units of 5 bp. WT, wild-type sequence; Ctrl, control construct in which a 5' restriction site was introduced (shaded). Panel C, CAT assay using the constructs in B. IL-1 induction was plotted as percent change.
**Fig. 2.** Transcription factor-induced bending at the NF-ELAM1/ATF and NF-κB I elements. Panel A, strategy used to generate permuted probes for band shift assays. The E-selectin elements NF-ELAM1/ATF or NF-κB I (box) were cloned into pBEND2, and probes cut out using different restriction endonucleases. Panel B, band shift assay using a short (E-selectin) or permuted NF-ELAM1/ATF elements and HUVEC nuclear extract. Competition was (C) with unlabeled ds EL1a/b (Table I; same as “short” probe) DNA or with the mutant (M) sequence EL1 mut a/b. F, free probe; n.s., nonspecific complex. Panel C, idev for the NF-κB I element. The E-selectin short probe was Table 1, and the same sequence was used in competitions (C). The mutant (M) competitions were with xBmut a/b. Panel D, plot (using data from B and C) of the $R_f$ of the various complexes versus recognition element position (their symmetry centers) in the probes. The dotted lines represent extrapolations.

A–E oligonucleotides (Table I) were phosphorylated, annealed pairwise (left overnight in a switched-off 90 °C water bath), and cloned into the MluI-digested, phosphatase-treated starting construct. All promoters were sequenced to determine copy number and orientation of the inserts. To test the promoter activities, HeLa cells were co-transfected with a CAT-reporter construct that contains multiple tetracycline-repressor binding sites in a minimal thymidine kinase promoter (23, 24).

**Cell Culture, Transformations, and CAT Assay**—These were all performed using previously described procedures (12). CAT chromatograms were scanned for radioactivity (using an AMBIS scanner), and percentage of CAT conversions were calculated. Values were averaged for at least two experiments and are expressed as percent change with respect to non-mutated or 5-bp shifted mutants (in the case of the 10-bp shifts).

**Band Shift Assays**—Probes for NF-κB- and ATF bending were made as follows. The polylinker repeats plus enhancer element were amplified by PCR from the pBend2-derivatives using oligonucleotides PCR BENDa and -b (Table I). The resulting fragments were phenol-treated,
**TABLE I**

**Sequences of oligonucleotides used in this study**

| Oligonucleotide | Sequence (5' to 3') |
|-----------------|---------------------|
| E-selectin NF-ELAM-I | ATA ACG CGT GGA TAT TCC CGG GAA AGT TTT TGG |
| E-selectin NF-KB I | ATA ACG CGT CTT AAA ATT ACA ATG ATG TCA GAA ACT CTG TCT C |
| E-selectin NF-ELAM-I +10bp | ATA ACG CGT GGA TAT TCC CAG CAT GGG AAA GAT TTG GGA TGG CAT TGG GGA |
| E-selectin NF-KB I +10bp | ATA ACG CGT TCT CCC CAT GCT TAA AAT TAC AAT GAT GTC AGA AAC ACT GTC TCC TCT TCA TGG A |
| E-selectin NF-ELAM-I -10bp | ATA ACG CGT TAT TCC CAG CAT GGG AAA GAT TTG GGA TGG CAT TGG GGA |
| E-selectin NF-KB I -10bp | ATA ACG CGT AAC TGA TCC CAT GGT AAC GAG ATG GAT GTC AGA AAC ACT GTC TCC TCT TCA TGG A |
| NF-ELAM-I +5a | ATA TGC GGC GCC TCT GAC ATC ATT TTT AAG CAT TGG |
| NF-KB I +5a | ATA TGC GGC GCC TCT GAC ATC ATT TTT AAG CAT TGG |
| NF-ELAM-I +10a | ATA ACG CGT TCT CCC CAT GCT TAA AAT TAC AAT GAT GTC AGA AAC ACT GTC TCC TCT TCA TGG A |
| NF-KB I +10a | ATA ACG CGT TCT CCC CAT GCT TAA AAT TAC AAT GAT GTC AGA AAC ACT GTC TCC TCT TCA TGG A |
| EL1 -5bp | ATA TGC GGC GCC TCT GAC ATC ATT TTT AAG CAT TGG |
| EL1 -10bp | ATA TGC GGC GCC TCT GAC ATC ATT TTT AAG CAT TGG |
| EL1-BENDa | TCA GTT TCT GAC ATC ATT GT |
| EL1-BENDb | TGG AAC ATG GTC AGA AA |
| EL1a | CAG AGT TTC TGA CAT TGT AAT TTT AAG C |
| EL1b | GCT TAA AAT TAC AAT GAT GTC AGA AAC TCT G |
| EL1mut a | CAG AGT TTC TGA CAT TGT AAT TTT AAG C |
| EL1mut b | GCT TAA AAT TAA CCG TCG TGA AGA AAC TCT G |
| PCR BEND2a | AGG CCG ATC ACG AGG CCC TTT CTT C |
| PCR BEND2b | ATA CAC CTT TGA TCA TGG CTT TAG C |
| B-BENDa | TCG ATA AGG AAA TCC CCA AT |
| B-BENDb | ATA TGA AAG GAA TCC CCA ATG GCA TCC |
| Curve-A: | CCG GCC TAG CTA AAA TGG CCT AAA ATG GCC CTA AAA TGG CCT AAA ATG GCC CTA AG |
| Curve-A: | CCG GCC TAG CTA AAA TGG CCT AAA ATG GCC CTA AAA TGG CCT AAA ATG GCC CTA AG |
| Curve-B: | CCG GCC TAG CTA AAA TGG CCT AAA ATG GCC CTA AAA TGG CCT AAA ATG GCC CTA AG |
| Curve-B: | CCG GCC TAG CTA AAA TGG CCT AAA ATG GCC CTA AAA TGG CCT AAA ATG GCC CTA AG |
| Curve-C: | CCG GCC TAG CTA AAA TGG CCT AAA ATG GCC CTA AAA TGG CCT AAA ATG GCC CTA AG |
| Curve-C: | CCG GCC TAG CTA AAA TGG CCT AAA ATG GCC CTA AAA TGG CCT AAA ATG GCC CTA AG |
| Curve-D: | CCG GCC TAG CTA AAA TGG CCT AAA ATG GCC CTA AAA TGG CCT AAA ATG GCC CTA AG |
| Curve-D: | CCG GCC TAG CTA AAA TGG CCT AAA ATG GCC CTA AAA TGG CCT AAA ATG GCC CTA AG |
| Curve-E: | CCG GCC TAG CTA AAA TGG CCT AAA ATG GCC CTA AAA TGG CCT AAA ATG GCC CTA AG |
| Curve-E: | CCG GCC TAG CTA AAA TGG CCT AAA ATG GCC CTA AAA TGG CCT AAA ATG GCC CTA AG |

**RESULTS**

Moving the E-selectin NF-ELAM-I/ATF or NF-κB II elements half-helix turns up- or downstream Periodically Affects Promoter Activity—A panel of E-selectin promoter mutants was constructed such that the NF-ELAM-I or NF-κB II elements were moved units of 5 bp (half of a DNA helix turn) up- or downstream. The resulting promoters (containing 383 bp of the upstream E-selectin sequence) were tested as CAT reporter constructs. Fig. 1A gives an overview of the proximal E-selectin promoter, and the transcription factors known to bind at the various DNA elements. Fig. 1B shows in detail how the promoter sequence was modified for each of the mutants. The construction procedure (see *Experimental Procedures*) required the insertion of unique restriction sites at the 5' end of the enhancer elements. This modification did not affect promoter activity (data not shown). Fig. 1C summarizes the results of testing the mutants in both primary endothelial (HUVEC) and established epithelial (HeLa) cells. In general, moving either DNA element 5 bp (half-helix turn) up- or downstream resulted in a strong reduction in promoter activity, whereas shifts of 10 bp in either direction partly or completely restored IL-1 inducibility. An exception to this rule was the NF-ELAM1/ATF element; moving it further upstream mainly resulted in a decrease of promoter activity.

All Factors Known to Bind the E-selectin Promoter Bend DNA—Bending of DNA targets has previously been established for TF-IID, various ATF members, HMG I(Y), and NF-κB. All of these have also been shown to bind the E-selectin promoter (summarized in Fig. 1A). We have previously shown that the E-selectin NF-κB I element is in fact a target for two het-

Cut with the different restriction enzymes, phosphorylase-treated, purified from a Metaphor agarose (FMC BioProducts) gel, end-labeled using γ-32P[ATP], and purified again on a 12% non-denaturing polyacrylamide gel (20). The total length of the probes is 137 bp for NF-ELAM-I and 139 bp for NF-κB II. Standard probe and nuclear extract preparation, and band shift assays were as described previously (20, 26, 28).

*Floting of Intrinsically Curved DNA*—Coordinates for the molecules in the plane where bending is maximal were calculated using the CURVATURE software (27, 28). The E-selectin promoter region corresponds to coordinates 169 to 81, the ds curve oligonucleotides were as in Table I. These coordinates were plotted using ORIGIN 3.0 (MicroCal) software.
FIG. 3. Replacement of the NF-ELAM1/ATF element by intrinsically bent DNA. Panel A, strategy: promoter constructs were made in which the NF-ELAM1/ATF element was block-mutated, and ds curved oligonucleotides were inserted upstream of the NF-κB II element. Panel B, conformation of the wild-type (WT) E-selectin promoter (−169 to −81) and the ds curve oligonucleotides. The E-selectin promoter and curve A were...
erodimeric complexes: one is the well known NF-κB1/rel-a heterodimer (nomenclature as in Ref. 29), the second form consists of NF-κB1 plus a 75-kDa polypeptide that is serologically related to rel-a. This latter complex is called NF-κB+ (30). We decided to investigate whether NF-κB+ and the multiple ATF-like factors binding at the NF-ELAM-1/ATF site bend DNA as well. For this purpose, NF-ELAM1 and NF-κB elements were separately cloned into vector pBend2 (31), between two direct polylinker repeats (see Fig. 2A). From this vector, probes for band shift experiments can be excised, in which the E-selectin promoter element is located at different positions within the DNA fragment (Fig. 2A, lower half). The results of these band shifts are shown in Fig. 2B for the NF-ELAM-1/ATF element, and in Fig. 2C for the NF-κB I element. The relative mobilities (R_p) for the various complexes have been plotted in Fig. 2D. Several complexes are obtained using the NF-ELAM-1 probe. At least for two of them, called NF-ELAM1a and -b (12), phasing results in a clear change in R_p. The band marked n.s. is not competed away by the short, unlabeled NF-ELAM-1 oligonucleotide (C lanes), and therefore does not represent factors binding the E-selectin promoter. Fig. 2C shows that the "short" E-selectin NF-κB probe (κ-a and -b in Table I) binds only NF-κB (first three lanes), whereas the same element embedded in a longer probe binds NF-κB+ as well. As described previously, NF-κB+ requires extra 3` DNA for binding in vitro, although the particular nucleotide sequence is not relevant (30). As Fig. 2, B, C, and D, clearly shows, NF-κB, NF-κB+, NF-ELAM1-a, and -b all bend DNA. The bending angles were calculated by fitting μ_p/μ_b in a standard curve for 4.5% polyacrylamide gels (32) and are plotted in Fig. 3B, C, and E) and IL-1 inducible (curves B, C, and E) and reached nearly wild-type activity levels (24). The activities imparted by the bent DNA fragments are highly dependent on their orientation (cf. A- and A+) and plane of bending (cf. D and E). Since the bent DNA-fragments are repetitive, and highly similar in sequence, it is very unlikely that the transcriptional enhancement seen for some of them results from binding of transcription factors. Moreover, band shift assays using the bent fragments as probes failed to yield specific DNA-protein complexes (data not shown).

Table II

| Transcription factor | μ_p/μ_b | Bending angle (°) |
|---------------------|---------|-------------------|
| NF-ELAM-1a          | 0.842   | 80°               |
| NF-ELAM-1b          | 0.834   | 86°               |
| NF-κB               | 0.838   | 83°               |
| NF-κB+              | 0.739   | 110°              |

DISCUSSION

Regulated promoter activity is the result of subtle interplay among a limited number of promoter elements. Nature seems to rely heavily on the use of combinatorial diversity of DNA elements, while using only a relatively limited set of different enhancers and transcription factors. One of the current challenges is to understand by what mechanisms these interactions take place (the problem was recently reviewed in Ref. 35). One important characteristic by which transcription factors synergistically cooperate is by direct protein-protein interaction. Thus, the triggering inducer of the E-selectin promoter, NF-κB, has been shown to interact with ATF-a, c-Jun (10, 36, 37), ATF-2 (38), TF-IID (39), and HMG I(Y) (35). Furthermore, direct protein-protein interactions have been demonstrated between HMG I(Y) and ATF-2 (38). Although these interactions are probably weaker than DNA-protein affinities, they may nevertheless assist in the formation of a functional pre-initiation complex.

A second mechanism by which DNA elements may combinatorially affect transcriptional activity is by structural alteration (bending or kinking) of the promoter (recently reviewed in Ref. 40). Protein-induced bending as a transcriptional switch has been well established in prokaryotes (e.g. see Refs. 41 and 42). An example in eukaryotes is E2F, whose association with the retinoblastoma gene product results both in reduction of the DNA bend it causes and its transcriptional activity (43).

We show here that the spacing of the IL-1 inducible E-selectin promoter elements is highly sensitive to up- or downstream shifts in a manner that suggests that the relative helical orientation is important. Although this type of result is usually only taken as evidence that protein-protein interactions are important for promoter activity, we decided to take a closer look at induced bending of the E-selectin promoter. The rationale is that phasing of DNA bends results in a change of the plane in which the angle is situated. As we have shown here, factors binding the NF-ELAM-1 and NF-κB sites also bend their DNA target. The bending angle determined by phasing analysis is highly dependent on polyacrylamide concentration, temperature, buffer system and gel run length (19). Nevertheless, the value we found for NF-κB (α = 83°) fits well with those reported in Schreck et al. (19). It is also clear that NF-κB+ (50°/75°) induces a sharper bend than NF-κB (50°/65°). The altered DNA-bending by the two NF-κB complexes is consistent with

plotted in the planes where their bending is maximal; the curve B-E fragments are plotted in planes whose angle with curve A is indicated. "Plus" and "minus" signs refer to orientation of the curve inserts ("plus" is as in Table I). Panel C, transcriptional activity of the E-selectin promoters carrying curve inserts (as shown in A).
p65 being responsible for bending in NF-κB (19). Since p65 is also responsible for NF-κB’s trans-activating potential (44), one may speculate that p50/p65 plays an important role in E-selectin promoter activity. We have previously shown that p50/p65 requires extra DNA contacts 3’ of the NF-κB binding site (30). Since the center of bending is the same for NF-κB and NF-κB* (Fig. 2D), this might mean that NF-κB* partially “wraps” the DNA around the 75-kDa subunit, resulting in increased apparent bending with conservation of its bending center.

Like the NF-κB-elements, the NF-ELAM site has imperfect symmetry (TGACATCA), and this site is a well conserved deviation from the symmetrical CREB site (TGATATCA). It is therefore likely that this site is mainly the target for heterodimeric bZIP factors. In this context it is interesting that ATF-2 homodimers hardly bend DNA, whereas ATF2Ic-Jun heterodimers do (17).

We have shown here that the NF-ELAM1/ATF element can be functionally replaced by intrinsically bent DNA. The NF-ELAM1/ATF element has no enhancer activity on its own, even when multimerized, but is able to stimulate neighboring ens-...