Mechanisms in the Transcriptional Regulation of Bradykinin B$_1$ Receptor Gene Expression

IDENTIFICATION OF A MINIMUM CELL-TYPE SPECIFIC ENHANCER*

(Received for publication, December 17, 1997, and in revised form, February 22, 1998)

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To investigate the mechanisms of bradykinin B$_1$ (BKB$_1$) receptor gene expression, transient DNA transfection analyses of human BKB$_1$ receptor gene promoter were performed in SV-40 transformed IMR90 cells. A positive regulatory element (PRE) located at position −604 to −448 base pair (bp) upstream of the transcription start site consistently exhibited, by far, the highest level of relative luciferase activity. A negative regulatory element, at position −682 to −604 bp, was able to completely ablate the function of the PRE. Transfection combined with deletion and mutation analyses illustrated that the PRE contains a classic, powerful enhancer. This enhancer was minimized to a 100-bp element at position −548 to −448 bp. A 78-bp fragment of negative regulatory element functioned as a silencer. Transient transfection of the enhancer construct, driven by heterologous herpes simplex thymidine kinase promoter, into a variety of cell types, showed that this enhancer presents a cell-type specific feature. In the characterization of the enhancer, motifs A (−548 to −532) and B (−483 to −477) were found to be essential for full enhancer activity. Motif D (−472 to −467) played a smaller role in enhancer activation. Gel shift and antibody supershift assays determined that an AP-1 factor binds with motif B. The nuclear protein which binds to motif A has yet to be identified. Both factors are the critical regulators for this enhancer activation.

Bradykinin (BK) receptors are members of the G-protein coupled receptor family (1). A large body of evidence points to the involvement of BK receptors in inflammation and shock leading to hypotension and pain (2, 3). Two subtypes of bradykinin receptor (BKB$_1$ and BKB$_2$) with variable ligand binding affinities and different agonists and antagonists have been characterized (2, 4–6). The BKB$_1$ receptors are generally only minimally expressed under normal physiological conditions. But functional BKB$_1$ receptors are rapidly induced under many inflammatory conditions such as arthritis, cystitis, UV irradiation, colitis and hyperalgesia, and inflammation (3, 7–11). However, these receptors are expressed only in a limited number of human tissues such as lung, colon, stomach, and bladder (7, 8). In vitro studies have implicated LPS, PMA, and various cytokines, such as interleukin-1β and interleukin-2, interleukin-8, and interferon-γ as effectors in the rapid induction of BKB$_1$ receptor expression (7, 8, 12–14).

The mechanism(s) leading to the rapid induction of this gene are unknown. Evidence suggests that the expression of BKB$_1$ receptors is regulated at both transcriptional and post-transcriptional levels (3, 7, 8, 14). The genomic sequence of the human BKB$_1$ receptor gene was recently reported by several groups including ours (6, 15, 16). So far neither detailed functional promoter structure nor the regulatory elements of this gene have been defined.

The aim of this study was to begin to characterize the functional promoter. We identified a powerful enhancer and certain crucial cis-acting regulatory elements of this gene. Initially, we used a transient transfection strategy to characterize the functional promoter and localize the important regulatory elements. By using deletion and mutation strategies, a minimum enhancer element and its regulatory sequence motifs were defined in the 5′-flanking region. Gel shift and antibody supershift procedures were then used to identify an AP-1 factor and another unknown factor as crucial transcriptional regulators of this enhancer.

EXPERIMENTAL PROTOCOLS

Plasmid Constructs—The BKB$_1$ 5′-flanking region was previously isolated as a λ genomic clone (6). By subcloning and sequencing we defined approximately a 1.8-kb fragment upstream of the transcription start site. Its nucleotide sequence was submitted to GenBank (access number U48827). A schematic representation of the pGL-BK-LUC constructs is shown in Fig. 1. The pGL-BKA-LUC was generated by using PCR to generate a 1.8-kb fragment from a subclone followed by cloning into the SacI and HindIII sites of pGL-3-Basic vector (Promega, Madison, WI) harboring a promoter segment from 1,748 to +89. The pGL-BKB-LUC was constructed by SmaI digestion of pGL-BKA-LUC to remove a 400-bp fragment. The other five 5′-end deleted constructs were generated by either standard restriction enzyme digestion or PCR and were cloned into the pGL-3-Basic vector. The exact end points of the deletions are presented in Fig. 1.

The putative enhancer (−604 to −362) element and its six deletion mutants (Fig. 5) were generated by PCR and subcloned into pGL-TK-LUC vector. This vector contains the luciferase gene driven by the herpes simplex virus-thymidine kinase (TK) promoter. The exact end points of the deletions and location are shown in Fig. 5. The oligonucleotide sequences and location used for enhancer mutagenesis are shown in Fig. 7A. These oligonucleotides were used in PCR or site-directed mutagenesis to generate four mutants of the enhancer. The constructs were subcloned into pGL-TK-LUC vector. The constructs containing different orientations and positions of the enhancer were generated with standard restriction enzyme digestion and cloning techniques.

All the oligonucleotides used in this study were synthesized either in
the DNA-Protein Core of our medical center using an ABI DNA synthesizer, model 394-08 or were purchased from Life Technologies, Inc. (Gaithersburg, MD). All of the PCR products were first cloned into the TA-cloning vector from Invitrogen Inc. (Carlsbad, CA) and then subcloned into other plasmids. All of the deletion and mutation constructs and their orientations were confirmed by the DNA-Protein Core of our medical center using an automatic DNA sequencer (Applied Biosystem Inc., Model 370A). The fidelity of the constructs was further confirmed with the use of restriction enzymes.

\[ [\text{3H}]{\text{des-Arg}}^{10}\text{kallidin} \]

Nonspecific binding was determined in the presence of 0.15% bovine serum albumin. The cells were solubilized with 0.25 M unlabeled [des-Arg10]kallidin. After 2 hours at 4 °C, the cells were washed three times with 1 ml of wash buffer (binding buffer supplemented with 0.15% bovine serum albumin). The cells were solubilized with 0.25 ml of 0.2% SDS and radioactivity determined in a 1011 LKB counter equipped with a luminescence detector.

For electrophoretic mobility shift assays, the protocol used was described previously (20). Briefly, 2–5 μg of nuclear extract was preincubated in the binding buffer with/without oligonucleotide competitors for 10 min at room temperature. The reaction mixture was then incubated with 1 μl of 32P-labeled probe for 20 min at room temperature. Samples were run on 5% acrylamide gels at 150 V and gels were dried and exposed to an x-ray film (Kodak). For supershift assays, 2 μg of AP-1 polyclonal antibody which recognizes Jun family protein (c-Jun, JunB, and JunD) or NF-1 polyclonal antibody reactive with all NF-1 isoforms (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with the nuclear extract reaction mixture for 45 min at room temperature before adding the probe.

RESULTS

**BKB1 Receptor Gene Promoter Is Regulated by Both Positive and Negative Elements**—The genomic structure of human BKB1 receptor including part of the 5′-flanking region has been reported recently (6, 15, 16). To delineate the sequences essential for transcription of the BKB1 receptor gene and identify important regulatory domains, we cloned approximately a 1.8-kb fragment of the 5′-flanking sequence from a λ-genomic clone (6). A series of six 5′-end deletion mutants were generated. One additional construct (pGL-BKB-LUC) was generated by a deletion between positions −1495 and −1092. These constructs were transfected into IMRSV cells. Reporter gene luciferase activities were measured. Several positive and negative regulatory regions were identified (Fig. 1). Two positive regions were found at nucleotide positions −730 to −682 and −604 to −448. Much lower luciferase activities were observed when
these regions were deleted. Three negative regulatory regions (position −1745 to −730, −682 to −604, and −448 to −384) were identified. Deletion of these regions resulted in a marked increase in luciferase activity. A strong positive element (PRE) located in position −604 to −448 proved of particular interest. This element produced a very large (over 34-fold) increase of reporter gene expression. A strong negative element (NRE) was located in the immediate upstream left of the PRE at position −682 to −604. This element completely inhibited the function of the PRE. These observations point to the presence of a dominant enhancer and a strong silencer in the 5′-flanking region of the inducible BKB1 receptor gene.

**The Expression Pattern of BKB1 Receptors in Different Cell Types**—Only a limited number of cell types and tissues have thus far been reported to express the BKB1 receptor (7, 8). Binding of [3H]des-Arg10-kallidin to IMR90, IMR95, HEK-293, Rat-1, and COS cells was performed to determine the expression of BKB1 receptor in these cell types. The results illustrated in Fig. 2 show that HEK-293, Rat-1, and COS cells do not exhibit endogenous BKB1 receptor binding. Whereas, IMR90 and IMR95 cells exhibit statistically significant endogenous BKB1 receptor binding.

**Cell-type Specific Feature of cis-Acting Regulatory Elements in the Promoter of Human BKB1 Receptor Gene**—To determine whether the regulatory elements in this gene promoter have cell-type specific features, construct pGL-BKE-LUC (containing PRE and NRE) and construct pGL-BKB-LUC (containing PRE only) was determined in several cell types with/without constitutive expression of BKB1 receptors. The relative luciferase activity (mean ± S.D.) from pGL-BKE-LUC was compared with the data from pGL-BKB-LUC (assigned a value of 1). Transient transfection experiments were performed in triplicate and repeated at least three times for each construct. Values shown are normalized with β-galactosidase activity and are expressed as relative luciferase activity.

**Regulation of Bradykinin B1 Gene Expression**

**Cell-type Specific Feature of the BKB1 Receptor Enhancer**—To determine whether the enhancer has cell-type specific function,
constructs with/without the minimal 100-bp enhancer, driven by TK promoter, were transfected into IMR90, IMRSV, HEK-293, Rat-1, and COS cells. Results shown in Fig. 6 proved very interesting. The enhancer fully activated the TK promoter in IMR90 and IMRSV cells. There was no enhancer activation in HEK-293, Rat-1, and COS cells. These results provide further evidence that there is a cell-type specific enhancer in the 5'-flanking region of the BKB1 receptor gene. The function of the enhancer correlates with the cellular expression of the BKB1 receptor.

**Dissection of the 100-bp Enhancer Element Identifies Multiple Positive Motifs Required for Full Enhancer Activity**—To characterize the regulatory motifs of the 100-bp enhancer element, a search for consensus sequences of transcription factors identified several potential nuclear factor-binding sites. Some of the common consensus sequences in the enhancer element are shown in Fig. 7A. An NF-1-like binding site (motif A) is located at the 5'-end of the enhancer. An AP-1 site (motif B) is located in the 3'-end of the enhancer. One PEA-3-binding site (motif C, PEA-3A) is adjacent to the 5'-end of motif A (NF-1-like site). Another PEA-3 site (motif D, PEA-3B) is located adjacent to motif B (AP-1 site) in the 3'-end of the enhancer. To determine whether any of these consensus sequences are essential for the function of the enhancer, four mutants were generated by site-directed mutagenesis using the enhancer as a template. The sense strand oligonucleotides used for the mutagenesis are shown in Fig. 7A. These mutant constructs were transfected into IMR90 to evaluate the enhancer activity. Results are shown in Fig. 7B. As compared with the original enhancer, less than 20% of enhancer activity remained when motif A (NF-1-like site, 546 to 534) was mutated. En, -nner activity was almost eliminated when motif B (AP-1 site, 485 to 477) was mutated. Moreover, further deletion at the 5'-end and mutation of motif C (PEA-3A site) did not show a meaningful change in reporter gene luciferase expression when compared with the mutant activity where the NF-1-like site was mutated. The 3'-end deletion and mutation of motif D (PEA-3B site) did bring the relative luciferase activity down more than half but did not abolish the enhancer activity. These observations suggest that motifs A, B, and D are required for full enhancer activity. Motifs A and B play a major role in enhancer activation.

**Specific DNA-Protein Interactions within Enhancer Region of Human BKB1 Receptor Gene in IMRSV Cells**—To examine the interaction of the crucial motifs, A and B, to interact with transcription factors, two probes, assigned as A (overlapping the motif A) and B (overlapping the motif B), were synthesized and used in the electrophoretic mobility shift assay. Electrophoretic mobility shift assay revealed a specific set of protein-DNA complexes when nuclear extracts prepared from IMRSV were incubated with 32P-labeled oligonucleotide probes A and B (Fig. 8, A and B). Two intense complexes, designated as complex R1 and R2, bound to oligonucleotide A. The specificity of these protein-DNA interactions was confirmed by increasing inhibition of binding in the presence of a 50- and 100-fold molar excess of unlabeled oligonucleotide A and no, or minimal, inhibition in the presence of excess of unlabeled unrelated consensus sequence such as SP-1, AP-3 (Stratagene). To further explore the specificity of the nuclear factor binding to probe A, an unlabeled mutant oligonucleotide (M-1), in which a NF-1-like site was mutated, was employed as competitor in the binding assay. As shown in Fig. 8A, the unlabeled mutated A competed as efficiently as the wild type A for the formation of the two specific complexes. This suggests that the mutated nucleotides are not crucial for the formation of the two specific complexes. For further confirmation, the NF-1 consensus oligonucleotide, CTF (from Stratagene), was used as competitor in the binding assay. The NF-1 consensus oligonucleotide failed to inhibit the formation of the two specific complexes. This observation suggests that the two specific complexes are not formed by an NF-1 factor.

Incubation of 32P-labeled probe B with nuclear extracts from IMRSV resulted in the formation of three protein-DNA complexes designated S1, S2, and S3 (Fig. 8B). The formation of these complexes was inhibited by the inclusion of a 50- and 100-fold excess of unlabeled oligonucleotide B. But the complex formation was only minimally inhibited at best in the presence of excess of unlabeled unrelated consensus sequence such as AP-1, CREB (Stratagene). In addition, the three complexes were all abolished by the unlabeled mutated oligonucleotide B (M-2). These results are in agreement with the electrophoretic mobility shift assay.
of excess of unrelated competitors such as consensus sequences SP-1, AP3, and CTF. On the other hand, the AP-1 consensus sequence totally inhibited the complex formation as efficiently as unlabeled oligonucleotide B. In addition, further confirmation also showed that unlabeled mutated oligonucleotide (M-3), in which the AP-1 site was mutated, failed to compete with probe B in the formation of specific complexes. These results suggest that oligonucleotide B contains an AP-1 site binding with a nuclear protein. To further determine whether any of the three specific complexes are generated by an AP-1 transcription factor binding with probe B, antibody supershift was performed using an AP-1 polyclonal antibody which recognizes the Jun family of proteins (c-Jun, JunB, and JunD). As shown in Fig. 8C, the AP-1 antibody supershifted the formation of complex S1, whereas NF-1 antibody was unable to supershift the S1 complex. These results suggest that the 3'-end of the

FIG. 6. BKB1 enhancer activity in different cell types. The relative luciferase activity (mean ± S.D.) of the enhancer construct pGL-Be100-TK-LUC compared with the value from enhancerless vector, pGL-TK-LUC (assigned a value of 1), was determined in several cell types with/without constitutive expression of BKB1 receptors. The cell types tested including: IMR90, IMRSV, HEK-293, Rat-1, and COS cells.

FIG. 7. A, the putative protein-binding sites in the human BKB1 enhancer element. At the top, the putative protein-binding regions are depicted in the enhancer element, and assigned as separate motifs, motif A contains the nucleotides of NF-1-like site, motif B contains the nucleotides of AP-1 site, motif C contains the nucleotides of PEA-3a site, and motif D contains the nucleotides of PEA-3b site. At the bottom, the putative nucleotide consensus sequences are shown using uppercase letters in the nucleotide sequences of the 100-bp minimum enhancer. The four mutant sequences located above and below the core enhancer sequences are the primers used in the mutagenesis of the enhancer element. The nucleotides chosen for mutagenesis in the enhancer are indicated by asterisks above or below those nucleotides, and the corresponding mutated nucleotides are shown by uppercase letters in the mutant sequences. Double stranded oligonucleotides from mutant 1 and mutant 3 were also used as competitors in the gel mobility shift assay. B, mutational analysis of the minimum enhancer activity. The wild type enhancer fragment and its mutated derivatives, in which either motif was mutated (blank boxes) or a combination of motif mutation and nucleotide deletion were used, were fused to the pGL-TK-LUC vector. The transient transfection in IMRSV cells was performed in triplicate in each experiment. Each experiment was repeated at least three times. The data presented are mean ± S.D. from one of those triplicate experiments. The level of activation is depicted as a percentage change with the level obtained from wild type enhancer construct (assigned a value of 100).
enhancer has an AP-1-binding site. The site binds to an AP-1 transcription factor likely to modulate transcription of the BKB1 receptor gene.

*Induction of Human BKB1 Receptor Gene Transcription—* LPS, TNF-α, PMA, and BK effectors have been determined to up-regulate BKB1 gene expression by our laboratory and others (7, 8, 14). To assess inducible features of the full-length 1.8-kb promoter, we transiently transfected IMRSV cells with the pGL-3 construct containing the 1.8-kb full-length BKB1 promoter. At 48 h after transfection the cells were treated with LPS, TNF-α, PMA, or BK for 4 h. The cell extracts were harvested and luciferase activity was determined. Results, as shown in Fig. 9, demonstrate that BK stimulated luciferase activity by more than 2-fold over basal with a p value of less than 0.01. LPS, TNF-α, or PMA showed some stimulation of luciferase activity. But the activation did not prove to be statistically significant.

**DISCUSSION**

The 5′-flanking regulatory region of the BKB1 receptor gene was studied previously in some detail in our laboratory (6). The 361-bp functional promoter showed the presence of a typical TATA box, SP-1, AP-1, cAMP-response element binding protein, and cAMP-response element consensus sequences. In the present study, we extended the 5′-flanking region to a 1.8-kb fragment to further characterize the function of the promoter and identify potential domains involved in transcriptional regulation of this gene. Chimeric constructs were generated and their promoter activities were examined in transiently transfected IMRSV cells. Several distinct regulatory regions were found to be important. A dominant region, PRE, proved to function as a powerful enhancer. An adjacent region, NRE, 78-bp upstream of the PRE, functioned as a silencer.

To determine whether the PRE is a classic enhancer, it was inserted into the pGL-3 vector driven by a TK promoter in various orientations and positionings. All of these constructs generated large increases in luciferase activity. To our surprise, the placement of the PRE at the 3′-end of the luciferase gene actually doubled the enhancer capability to activate when compared with the constructs where the PRE was located at the 5′-end of the luciferase gene. It is unclear why we are finding this considerably higher induction of the reporter gene when the PRE is located at the 3′-end of the luciferase gene. However, interestingly, recently reported studies indicate that, in the presence of an enhancer, genes must exist in a supercoiled, looped, conformation to be actively transcribed (21, 22). Thus protein-protein interactions in vitro between a distal enhancer and a proximal promoter may be favored in a supercoiled DNA because of topological constraints (21, 22). The placement of the enhancer at the 3′-end of the luciferase gene may be favoring protein-protein interaction between transcription factors associated with the BKB1 enhancer and the TK promoter.

Deletion studies showed that the nucleotide sequences in the 5′- and 3′-end of the enhancer element are critical for full enhancer activation. Computer analysis of the consensus sequences in the enhancer region found a few common transcription factor-binding sites such as an NF-1-like site, an AP-1 site, and two PEA-3 sites located in the 5′- and 3′-ends of the enhancer element. Mutation and deletion studies focused on these binding sites. Mutation of motif A (NF-1-like site) of the enhancer resulted in a greater than 80% reduction of relative luciferase activity in IMRSV cells, illustrating that motif A is
crucial to the enhancer. However, the NF-1 consensus sequence failed to compete with probe A in the formation of the complex. Also, a mutant oligonucleotide of the NF-1-like sequence competed with probe A in the formation of this complex. These data indicate that this complex is not binding to a classic NF-1 factor. Further studies are necessary to identify this putative factor.

Mutation within motif B (AP-1) basically abolished the enhancer activity. This motif was then confirmed to bind an AP-1 factor by gel mobility supershift assay. These results in sum suggest that the AP-1 motif plays a dominant role in the transcriptional activation of the BKB1 receptor gene. Mutation of motif D (PEA-3B) reduced the enhancer activity by 38% of the wild type. Whereas, mutation of motif C (PEA-3C) of the enhancer did not show a meaningful effect on enhancer activity. The partial inhibition by PEA-3B mutant suggests that PEA-3B plays a role in the transactivation of the enhancer. Further studies are needed to determine whether the PEA-3B element plays a co-factor role and cooperates with other factors, especially with motif A, to activate the enhancer in response to effectors.

AP-1 and PEA-3 sites have been shown to interact and cooperate to activate basal and inducible expression of the metalloprotease (MMPs) gene family, tissue inhibitor of metalloproteinases gene family and the urokinase-type plasminogen activator gene (23–29). Notably, the BKB1 receptor gene shares certain similarities with the MMPs gene family. For example, both are inducible genes, which are up-regulated by effectors such as cytokines and growth factors (3, 7, 8, 12–14, 23). Both are rapidly induced during inflammation (3, 7, 8, 23). The two exhibit a high degree of similarity in their cis-acting regulatory elements in the 5′-flanking region, especially in the positioning of AP-1 and PEA-3 sites (23). Both genes have PEA-3 elements adjacent to the proximal AP-1 site and/or more distal AP-1 sites (23). Both genes demonstrate cell-type specific expression (23). The AP-1 and PEA-3 sites have been shown to play an important collaborative role in cell-type specific expression in the MMPs gene family (23). This coordinate and cell-type specific expression by AP-1 and PEA-3 is also found in the human urokinase-type plasminogen activator gene (24, 28, 29). In the present study, strong luciferase activity of BKB1 enhancer were observed in IMR90 and IMR9V cells, but not in HEK-293, Rat-1, or COS cells, illustrating the cell-type specific feature of this enhancer.

Furthermore, both the BKB1 and MMPs genes are induced by PMA stimulation (14, 23). The AP-1 and PEA-3 have been demonstrated as the responsive elements to PMA in the MMPs gene family and in the collagenase gene (23, 26, 27). On the other hand, NF-κB has also been reported to be involved in the activation by PMA in a number of other genes such as granulocyte-macrophage colony stimulating factor, urokinase-type plasminogen activator, interleukin-1β, and intercellular adhesion molecule-1 genes (30–33). Furthermore, NF-κB and AP-1 have been reported to synergistically transactivate the expression of the granulocyte-macrophage colony stimulating factor gene (30). We recently reported that PMA increases the steady state mRNA level of BKB1 receptor gene and consequently induces an increase in [des-Arg^{10}]kallidin binding. We found that the NF-κB activation inhibitor, pyrrolidinedithiocarbamate, inhibited the PMA induced increase in mRNA level of the BKB1 gene in IMR90 cells (14). AP-1, NF-κB, and PEA-3 sites have been located in the promoter of BKB1 gene by computer analysis. However, PMA did not elicit significant changes in luciferase activity in IMR95 transiently transfected with the 1.8-kb full-length BKB1 promoter. DeClerck et al. (25) reported that the promoter of human tissue inhibitor of metalloproteinases-2 gene failed to respond to PMA stimulation, despite the presence of AP-1 sites in the promoter region. They concluded that the positioning of the AP-1 site is crucial in response to PMA stimulation (25).

In the induction study of this gene promoter, BK generated more than a 2-fold increase of luciferase activity, but LPS, TNF-α, and PMA did not show significant increase of the luciferase activity compared with basal luciferase levels. These data suggest that different stimulators require different sizes of promoter or different regulatory elements to induce the transcription of the gene. Thus, a larger promoter construct, longer than 1.8 kb may be required to obtain significant inducibility by LPS, TNF-α, and PMA stimulation. Another possibility is that the differences in signal transduction between the native promoter, with the target gene in vivo, and the chimeric promoter, transiently transfected and linked to a luciferase gene, may be critical to the insensitivity in response to LPS, TNF-α, and PMA stimulation by the transfected BKB1 promoter.

Under normal, steady state conditions, the expression of this gene is tightly regulated by its native promoter, enhancer, and other regulatory elements. Results from transient transfection in IMR90 and IMR9V cells indicate that the expression of reporter gene luciferase is heavily inhibited by the upstream silencer of BKB1 receptor gene, and can be induced by removing the silencer from the immediate upstream left of the enhancer. This observation is entirely consistent with the minimal expression of the BKB1 receptor under normal physiological conditions, and rapidly inducible expression during inflammation. It provides a distinct transactivation model for expression and up-regulation of this gene by extracellular effectors.

In summary, we have characterized the functional promoter region and identified a cell-type specific enhancer in the 5′-flanking region of the human BKB1 receptor gene. An AP-1 transcription factor and another, unknown, factor appear crucial for full enhancer activity. We are also finding that a silencer directly suppresses the enhancer activity in IMR90 and IMR9V. This study paves the way for further analysis of the mechanisms involved in the transcriptional regulation of a gene which is rapidly induced under pathological conditions such as toxic sepsis which result in inflammation, hypotension, and pain.

Acknowledgments—We thank Ned Rich and his staff at DNA/Protein Analysis Core for their expertise in sequencing and oligonucleotide synthesis, and Dr. Gregory N. Prado for expert technical assistance in binding assay.

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