NF-IL6 (C/EBPβ) Vigorously Activates *il1b* Gene Expression via a Spi-1 (PU.1) Protein-Protein Tether*

Two classes of transcription factors, ETS and bZIP, stand out as key mediators of monocyte commitment and differentiation. The ETS domain factor Spi-1 (also called PU.1) and the bZIP factor NF-IL6 (also called C/EBPβ) have been shown to be involved in the transcriptional regulation of interleukin-1β gene (*il1b*) and other monocyte-specific genes. We now show that these two factors strongly cooperate on the *il1b* core promoter in the absence of direct NF-IL6 binding to DNA. Transient transfection assays, using mutated *il1b* core promoters, showed that the Spi-1, but not the NF-IL6, binding site is absolutely required for functional cooperativity. Furthermore, the NF-IL6 transactivation domain (TAD) is functionally indispensable and more critical than that of Spi-1. Additionally, TAD-deficient NF-IL6 functions as a dominant negative for Spi-1-mediated activation, suggesting the involvement of the bZIP DNA binding domain. This is supported by the demonstration of *in vitro* interaction between the NF-IL6 bZIP and Spi-1 winged helix-turn-helix (wHTH) DNA binding domains, arguing that NF-IL6 vigorously activates the *il1b* core promoter via protein-tethered transactivation mediated by Spi-1.

Myeloid lineage differentiation and the expression of activated monocyte/macrophage genes, such as the gene encoding interleukin 1β (IL-1β), depend upon the activity of at least two key transcriptional regulatory factors. One of these is NF-IL6 (also called C/EBPβ, NF-M, AGP/EBP, LAP, IL6-DBP, and CRP2) (1–6), which is constitutively expressed in resting monocytes and immediately activated by translocation from cytoplasm to nucleus by agents such as lipopolysaccharide, phorbol myristate acetate (PMA), and IL-6. NF-IL6 is a bZIP transcription factor that belongs to the C/EBP family of proteins (1, 3, 7). Recent studies have shown that NF-IL6 is capable of synergistically cooperating with other transcription factors, including NF-xB (8), Spi-1 (9), and GATA-1 (10). In these cases, the binding of NF-IL6 and its partners to their recognition sites in the promoters is required for the functional cooperativity. A second factor, Spi-1/PU.1, is a winged helix-turn-helix (wHTH) transcription factor that belongs to the ETS family of proteins. Spi-1 expression is primarily restricted to myeloid cells, whereas NF-IL6 is more broadly expressed. The importance of C/EBP factors and Spi-1 in myeloid cells emphasizes the need to understand the mechanisms regulating their functions.

IL-1β is an important inflammatory and immunoregulatory cytokine expressed by primarily activated monocytes/macrophages in response to a variety of stimuli, including lipopolysaccharide, PMA, IL-1β, and other cytokines (11). Uncovering the mechanisms that drive the expression of *il1b* will elucidate the events of normal myeloid commitment and differentiation as well as the dysregulation of gene expression that leads to inflammatory diseases. Zhang and Rom (12) reported that the -131/+12 region of the *il1b* promoter contained two NF-IL6 binding elements, located at positions -90/-82 and -41/-33. While the importance of the more upstream NF-IL6 site has been established (13), the function of the -41/-33 is poorly defined. This site overlaps the 3′-end of a Spi-1 binding site at -50/-39 by 3 base pairs. It was shown that in lipopolysaccharide-simulated macrophage cells, Spi-1, but not NF-IL6, is a predominant protein factor bound to this overlapping sequence (13, 14) in the -59/+12 region of the *il1b* core promoter. These data argue that the Spi-1, but not the overlapping NF-IL6 binding element, is required for maximal activation of the *il1b* core promoter.

There are accumulating evidence showing that Spi-1 and C/EBP family factors are important for the regulation of many genes involved in immunity and hematopoiesis, such as macrophage colony-stimulating factor receptor (15) and neutrophil elastase (16), in addition to the IL-1β gene. However, the mechanism responsible for the functional cooperativity is still poorly understood. Many ETS target sites are found adjacent to binding sites for other protein factors, which appear to functionally cooperate. The most frequently reported type of composite site involves cooperative interactions between ETS proteins and bZIP factors (17, 18), such as the interaction between the Spi-1 wHTH ETS DNA binding domain and the NF-IL6β (C/EBPβ) leucine zipper region (17). In transient expression assays, using an artificial promoter containing adjacent Spi-1 and NF-IL6β sites, Nagulapalli *et al.* (17) observed that Spi-1 and NF-IL6β could functionally cooperate to activate transcription. However, the combined roles of NF-IL6β and Spi-1 in naturally occurring promoters have not yet been reported. In most cases,
the association of another factor with Spi-1 results in strong synergistic activation of target gene. A recent example is c-Jun, which acts as a Spi-1 coactivator on the promoters of myeloid genes coding for macrophage scavenger receptor (19), and macrophage colony-stimulating factor receptor (20).

The close proximity of the Spi-1 and the putative NF-IL6 sites does not seem to be fortuitous. Even though the putative NF-IL6 site at positions −41−33 seemed to be not critical as a transcription factor binding site, as suggested by Buras’ in vitro data (14), we still investigated the possible functional involvement of NF-IL6 in the regulation of the il1b core promoter. The study described here demonstrates that NF-IL6 strongly cooperates with Spi-1 to activate the il1b core promoter (−59/+12), in which the integrity of the Spi-1 binding site, but not the putative NF-IL6 binding site, is critical for the synergy. In addition, the functional cooperativity between Spi-1 and NF-IL6 definitely requires the transcriptional domain (TAD) of NF-IL6, but not those of Spi-1. Spi-1 seems to act as an anchor, which tethers NF-IL6 to the il1b core promoter to exert activation, without NF-IL6 binding to its cognate binding site. This mechanism, which we have called protein-tethered transactivation (PTT) (20, 21), may be more widely used in gene activation than is presently appreciated.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells (Strain S3) were cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum and 0.5% penicillin-streptomycin as described previously (21).

Plasmid Constructions—The human il1b core promoter region (−59/+12) and its mutants were generated by polymerase chain reaction and inserted into pGEM3-Basic vector (Promega) at MluI and BgII sites to construct promoter-reporter plasmids. The pRc/CMV plasmids expressing the full-length Spi-1 and a series of Spi-1 deletion mutants were constructed as described previously (21) (22). Expression vectors for the full-length NF-IL6 (pcDNA3.1-NF-IL6) and a truncated NF-IL6 with an internal deletion between the two SpiI restriction sites (pcDNA1-NF-IL6ΔSpi) were constructed by inserting the NF-IL6 cDNAs (23) into expression vectors pcDNA3.1 or pcDNA1 (Invitrogen). GST fusion constructs containing various Spi-1 motifs were made as described previously (21). GST-NF-IL6, GST-NF-IL6ΔSpi, and GST-bZIP fusion protein expression vectors were constructed by inserting full-length, truncated ΔSpi NF-IL6 coding region, and a polyclonal antibody chain reaction- amplified fragment encoding the bZIP region of NF-IL6 from amino acids 269−345, into pGEX-2T (Amersham Pharmacia Biotech) at BamHI and EcoRI sites.

Transfections and Luciferase Assays—HeLa S3 cells were plated in 24-well plates 24 h before transfection. A total of 1 µg plasmid DNA, including 0.5 µg of reporter, 0.5 µg of expression vector, and 0.3 µg of pCMV-Sport-gal (Life Technologies, Inc.), except as noted, was transfected into the cells using DOTAP transfection reagent (Roche Molecular Biochemicals GmbH) as described previously (21). After incubation for 24 h, cells were stimulated with 50 ng/ml PMA (Sigma) for 20 h. The cells were then harvested and lysed in 150 μl of cell culture lysis reagent (Promega). The lysates were assayed for luciferase activity using the Promega luciferase assay kit.

Expression and Purification of GST Fusion Protein—Glutathione S-transferase fusion proteins were prepared by standard procedures as described previously (21). Equivalent amounts of GST fusion proteins (as determined by Bio-Rad and confirmed by Coomassie Blue staining) were bound to 50 µl of glutathione-Sepharose beads by incubation in a total volume of 500 µl of NETN (20 mM Tris chloride, pH 8.0, 1 mM EDTA, 50 mM NaCl, 3 mM MgCl2, 33 ng/ml poly[dI-dC], 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) and incubated for 5 min at 4 °C. The beads were then incubated for 20 min at 4 °C with 200,000 cpm of 32P-labeled wild-type probe corresponding to the il1b promoter sequence between −56 and +21 or the same probe with the putative NF-IL6 site mutated. The beads were washed twice with the binding buffer containing 10 ng/ml poly[dI-dC] and 0.5% Nonidet P-40. Specific binding was assayed by Cerenkov counting of the protein-DNA complex on the glutathione-Sepharose beads.

Electrophoretic Mobility Shift Assays—Double-stranded oligonucleotides spanning the overlapping Spi-1 and NF-IL6 sites (il1b promoter region from −56 to −21) were synthesized and labeled by using DNA polymerase Klenow fragment in the presence of [α-32P]dATP and [α-32P]dGTP. EMSAs were carried out by incubating 0.5 µl in vitro translated Spi-1 or NF-IL6 protein (TNT T7 coupled reticulocyte lysate system, Promega) with 10,000 cpm of the wild-type il1b probe, or the probe carrying mutations in either the Spi-1 site or the NF-IL6 site, under binding conditions of 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 3.3 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol with 1 µg of poly[dI-dC] in a final volume of 15 µl. The binding reactions were performed on ice for 20 min and then subjected to electrophoresis on 4% nondenaturing low ionic strength polyacrylamide gels using 0.5 × TBE buffer (TBE, 45 mM Tris borate, pH 8.3, and 1 mM EDTA). The gels were then dried and analyzed by autoradiography.

RESULTS

NF-IL6 Strongly Cooperates with Spi-1 to Activate the (−59/+12) il1b Core Promoter—We and others (12–14) have previously reported that the −59/+12 il1b core promoter contains a Spi-1 (−50/−39) and an NF-IL6 (−41/−33) binding site. To verify that Spi-1 and NF-IL6 could transactivate the il1b core promoter, we performed transient cotransfection assays. A luciferase reporter plasmid containing the il1b core promoter was cotransfected into Spi-1-deficient HeLa S3 cells (25) along with plasmids either expressing Spi-1, NF-IL6, or both (Spi-1+NF-IL6). The cells were stimulated with PMA 20 h before luciferase assays to activate the NF-IL6 protein. As shown in Fig. 1,
NF-IL6 alone only had a minimal effect, whereas Spi-1 stimulated activity by about 20-fold. However, NF-IL6 together with Spi-1 activated the \textit{il1b} core promoter by about 380-fold, suggesting a strong cooperativity between these two factors.

**Mutation of the Spi-1 Site, but Not the NF-IL6 Site, Abolishes Cooperative Activation**—To determine whether the cooperative activation of the \textit{il1b} core promoter by Spi-1 and NF-IL6 depends on their binding sites, site-directed mutagenesis was used to introduce multiple nucleotide sequence substitutions into the Spi-1 (mut Spi-1), NF-IL6 (mut NF-IL6), or both (mut both) binding sites, without changing the nucleotides in the overlapping region (Fig. 2). In agreement with previous studies (13), mutation of the Spi-1 binding site completely prevented activation by Spi-1, either alone or with NF-IL6 (Fig. 1). Transfection of NF-IL6 alone did not affect the promoter activity, even when the overlapping Spi-1 site was disrupted. A promoter containing an intact Spi-1 site and either an intact or mutated NF-IL6 site (WT or mut NF-IL6) supported activation by Spi-1 alone. Importantly, a promoter containing an intact Spi-1, but a mutated NF-IL6 site (mut NF-IL6) retained a majority of the synergistic activation in the presence of both Spi-1 and NF-IL6 (Fig. 1). Consequently, the Spi-1 binding site, but not the NF-IL6 binding site, is critical for the cooperative transactivation of this promoter by Spi-1 and NF-IL6.

Using the wild-type \textit{il1b} probe, containing both the Spi-1 and NF-IL6 sites (Fig. 3A), \textit{in vitro} translated Spi-1 can bind avidly to the probe in EMSA (lane 2), as suggested by previous reports (13, 14). Mutations in the core Spi-1 recognition sequence (AGAA to CTAA) abolished Spi-1 binding (lane 3). Due to the weak equilibrium binding observed by EMSA between NF-IL6 and the \textit{il1b} probe (~36/t1) (data not shown), we employed a GST-1-hybrid assay, capable of detecting complexes with either low affinity or high decay rates (21). Fig. 3B shows that a GST-bZIP fusion protein containing the NF-IL6 bZIP DNA-binding domain could bind to the wild-type probe about six times better than the binding between GST control protein and the probe. As expected, the substitution of 4 nucleotides in the NF-IL6 consensus region prevented the probe from being recognized by NF-IL6.

**The NF-IL6 TAD Is Indispensable for Functional Cooperativity, while Spi-1 TADs Only Partly Contribute**—NF-IL6 is a 345-amino acid protein with a COOH-terminal basic leucine zipper structure (bZIP) that binds to DNA. The TAD of NF-IL6 has been reported to bind directly to CBP/p300 (26). The extreme amino terminus of NF-IL6 is capable of recruiting SWI/SNF chromatin-remodeling complex and activating endogenous target genes in concert with the TAD (27). Between the TAD and the bZIP domains reside regulatory domains, which are involved in intramolecular interactions that inhibit transactivation and DNA binding when NF-IL6 is not activated by phosphorylation (28, 29) (Fig. 4A). Spi-1 possesses a COOH-terminal ETS wHTH domain that is involved in both DNA binding as well as protein-protein interactions involving AP-1 family members, NF-IL6 (C/EBPb), and other proteins (17, 18, 20, 30). The amino-terminal 170 amino acids of Spi-1 contains three independent transcriptional activation domains: a glu-

![Fig. 2. Human \textit{il1b} promoter mutants. Shown are the locations and identities of mutations used in this study (indicated by arrows). Numbering is relative to the transcription initiation site. Sequences conforming to Spi-1 and NF-IL6 consensus recognition sites are labeled.](Image 74x627 to 272x729)

![Fig. 3. Mutations within the \textit{il1b} probe abolish protein binding by Spi-1 and NF-IL6. A, \textit{in vitro} translated Spi-1 (lanes 2 and 3) were incubated with the \textit{il1b} probe (between nucleotides 56 and 21) in EMSA. Also, \textit{in vitro} translated Spi-1 was incubated with the same probe containing mutations in the core Spi-1 recognition region (AGAA to CTAA) in EMSA (lane 3). B, the GST-1-hybrid assay was used to detect weak protein-DNA interaction between NF-IL6 bZIP and the \textit{il1b} probe. GST and GST-bZIP fusion proteins immobilized on glutathione-Sepharose were tested for DNA binding activity using either wild type (\textit{wt}) \textit{il1b} probe (between nucleotides 56 and 21) or probe containing mutations in the putative NF-IL6 site (mNF-IL6). The results are the relative binding affinity with the cpm of mNF-IL6 probe bound to GST-bZIP set as 1. Error bars represent the S.E. in results from three repetitions.](Image 341x166 to 520x362)

![Fig. 4. NF-IL6 with a disrupted TAD cannot functionally cooperate with Spi-1. A, schematic representation of NF-IL6 protein structure. Functional domains, including a regulatory region (Reg) that inhibits the TAD and bZIP domains via intramolecular interaction, are shown. Also shown is the dominant negative NF-IL6 mutant (NF-IL6\textit{bZIP}). B, wild-type \textit{il1b} (59/12)-luciferase reporter plasmid was transfected into HeLa S3 cells in the presence of vectors expressing wild-type Spi-1 plus either mutant or wild-type NF-IL6. Activities are presented as in Fig. 1.](Image 365x504 to 498x729)
have shown that the activation of the il1b Core Promoter in HeLa Cells—Previously we binding specifically to DNA (13). expression of the various Spi-1 derivatives in HeLa cells with arguing for its dispensability. We have previously shown that Spi-1 alone activated the il1b NF-IL6-dependent activation (23) of the IL6. Deletion of the PEST region increased Spi-1 activation of 100, D

After incubation, the beads were intensively washed, and bound proteins were resolved by SDS-PAGE. As shown in Fig. 7A, NF-IL6 binds directly to full-length Spi-1 (lane 2), but not to the GST control (lane 1). More specifically, only the DNA binding domain of Spi-1 (amino acids 171–272) (lane 3) is required to mediate the interaction with NF-IL6. The GST fusion construct containing amino acids 202–254 (lane 4) bound to NF-IL6 as well as the full-length protein. The 202–254 region was further dissected into two pieces, which were also fused to GST (the 243–254 and 202–242 constructs, lanes 5 and 6). Both of these fusion proteins were capable of weakly binding to NF-IL6. It is possible that regions containing both the ETS and bZIP domains—It has been shown by protein-protein interactions with the lymphoid-specific coactivator NF-EM5/Pip/TRF-4 (33, 34) (Fig. 5A).

To determine the domains of Spi-1 and NF-IL6 required for transcriptional cooperativity, we assayed deletion mutations of both proteins in transient transfection assays. First, vectors coding for full-length NF-IL6 and a truncation (∆Spi1) that can bind to DNA, but lacks the TAD and a portion of the regulatory region, were co-expressed with full-length Spi-1 and assayed for il1b core promoter reporter activity in HeLa cells. Western analysis showed that both the full-length and the truncated NF-IL6 (∆Spi1) were correctly expressed by the transfected plasmids in HeLa cells (data not shown). The truncated NF-IL6 could not synergize with Spi-1 (Fig. 4B). Unlike the observations with NF-IL6, the Spi-1 mutant constructs (Fig. 5, A and B) with deletions of either the Q domain (∆Q), or the Q domain together with the NH2-terminal TBP binding domain (∆100, ∆NN) retained significant ability to cooperate with NF-IL6. Deletion of the PEST region increased Spi-1 activation of the il1b core promoter and its ability to synergize with NF-IL6, arguing for its dispensability. We have previously shown that expression of the various Spi-1 derivatives in HeLa cells with these vectors yielded comparable levels of proteins capable of binding specifically to DNA (13).

**Dominant Negative NF-IL6 Represses Spi-1-dependent Activation of the il1b Core Promoter in HeLa Cells**—Previously we have shown that the ∆Spi1 truncated NF-IL6 could antagonize NF-IL6-dependent activation (23) of the il1b upstream inducible enhancer (UIS), by competing with the wild-type endogenous NF-IL6 for a specific NF-IL6 binding site. We have transfected this dominant negative NF-IL6 mutant into HeLa cells in either the presence or absence of Spi-1. As shown in Fig. 6, Spi-1 alone activated the il1b core promoter. Strikingly, co-
length NF-IL6 (lane 2), the mutant ΔSpl NF-IL6 with the deletion of the region from amino acids 41–205 binds to the Spi-1 ETS domain (lane 4). It is noteworthy that deletion of the amino-terminal 268 amino acids of NF-IL6, leaving only the bZIP DNA binding domain, dramatically increased the binding affinity observed in the GST pull-down assay (lane 6). It was reported that, in the absence of activation, one of two regulatory elements (RD2, Fig. 4) from rat NF-IL6 could inhibit DNA binding by intramolecular interaction, whereas the other element (RD1) similarly inhibited transactivation (28, 29). Our data suggest that the RD2 domain, specifically the region from aa 206 to 268, may also prevent NF-IL6 from interacting with its cofactors.

In a reciprocal assay, 35S-labeled Spi-1 ETS domain (from amino acids 171–272) was prepared by in vitro transcription and translation and incubated with GST-bZIP fusion protein containing the bZIP region of NF-IL6 from amino acids 269–345. As expected, the labeled Spi-1 wHTH domain bound strongly to GST-bZIP, but not to GST alone (Fig. 7C).

**DISCUSSION**

The expression of il1b is regulated by two independent elements, an upstream inducible sequence (the UIS enhancer) and a cell type-specific promoter element (37). Although strong enhancer-dependent activity depends upon a long promoter extending from –131 to +12 (13), weaker enhancer-independent activity can be detected with the shorter –59/+12 promoter. Moreover, we have recently shown that the Spi-1 binding site located at position –50/–39 is responsible for mediating transactivation of il1b expression by cytomegalovirus IE2 protein, which eliminates the need for the otherwise essential upstream enhancer (21).

In this report, we have demonstrated that NF-IL6 dramatically cooperates with Spi-1 to activate the il1b core promoter, where the Spi-1 binding site, but not the putative NF-IL6 site, is critical. Although the Spi-1 recognition site is sufficiently important that mutations leading to a complete loss of Spi-1 binding result in a total loss of promoter activity in the presence of cotransfected Spi-1 and NF-IL6, deletion of the transcription activation domains of Spi-1 results in only a partial loss of its ability to functionally cooperate with NF-IL6. In contrast, the deletion of the NF-IL6 transactivation domain (aa 41–205) completely abolishes its ability to synergize with Spi-1 on the il1b core promoter. Physical interaction between the Spi-1 wHTH and the NF-IL6 bZIP DNA binding domains provides the basis for our model. In this model, the Spi-1 wHTH domain functions to recognize a specific site in the il1b core promoter and tether NF-IL6, which contains an efficient transcription activation domain, which, unlike those of Spi-1, is able to strongly activate il1b expression (Fig. 8). This is distinct from an earlier report of NF-IL6 cooperativity with glucocorticoid receptor, in which the NF-IL6 played a TAD-independent and indirect role (38). It should be noted that we could not detect a reproducible ternary complex (data not shown), involving NF-IL6, Spi-1, and DNA using either EMSA or a more sensitive GST-based two-hybrid approach (21), suggesting a tenuous interaction. Others have also attempted to detect ternary complexes involving NF-IL6 and have failed (38, 39), supporting this conclusion.

Our model is supported by two facts. First, cotransfection of NF-IL6 expression vector significantly increased the ability of both Spi-1ΔTAD and Q TADs and Spi-1ΔPEST (lacking the PEST region) to activate the il1b core promoter (Fig. 5). Second, although the dominant negative NF-IL6 was not able to repress the Spi-1-independent activity of the il1b core promoter in NF-IL6-expressing HeLa cells (22), it antagonized the Spi-1-mediated activation of the same promoter in a dose-dependent manner. This is consistent with the notion that Spi-1 activation of the il1b core promoter is mediated by NF-IL6 and that the putative overlapping NF-IL6 site is not functional. GST pull-down assays demonstrate that both NF-IL6ΔSpl and the full-length protein interact with Spi-1 at a similar level (Fig. 7), implying that NF-IL6ΔSpl may compete with endogenous NF-IL6 for interaction with Spi-1. It has been reported that Spi-1 without the TBP and Q TADs can activate transcription by playing an architectural role in interaction with NF-EM5/Pip/IRF-4 mediated by the PEST region (40).
Recently, we have shown that functional cooperativity between Spi-1 and the cytomegalovirus IE2 transcription factor does not require any Spi-1 TADs (including PEST) for activity (21). Our data now provide a new example in which the Spi-1 wTTH functions both to bind DNA and to tether a non-viral transcriptional factor containing a more potent TAD.

The mechanism by which the il1b UIS is integrated into the core promoter has always been a puzzle. We have reported that the UIS sequence between −3134 and −2729 contains two NF-IL6 binding sites (11, 23). Also we have shown that the −131/−59, which contains an additional Spi-1 binding site, is critical for enhancer activity (13). Our results now suggest the possibility that factors bound to the UIS, including LIL-Stat, CREB, and NF-IL6 (23), may be tethered to the proximity of the transcriptional initiation machinery through NF-IL6-Spi-1 interactions (Fig. 8). However, carefully designed experiments are needed to confirm this speculation.

In this report, we have shown that NF-IL6, which is abundant in myeloid cells (41), strongly synergizes with Spi-1 on the il1b core promoter via PTT (21) in transient transfection assays using Spi-1-deficient HeLa cells. This suggests that PTT also functions in IL-1β-dependent transcription, we have recently demonstrated that the viral protein PTT also functions both to bind DNA and to tether a non-viral transcription factor containing a potent TAD.

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