**Interleukin-6 and Leukemia Inhibitory Factor Induction of JunB Is Regulated by Distinct Cell Type-specific Cis-acting Elements**

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Interleukin (IL)-6 plays an important role in a wide range of biological activities, including differentiation of murine M1 myeloid leukemia cells into mature macrophages. At the onset of M1 differentiation, a set of myeloid differentiation primary response (MyD) genes are induced, including the proto-oncogene for JunB. In order to examine the molecular nature of the mechanisms by which IL-6 activates the immediate early expression of MyD genes, JunB was used as a paradigm. A novel IL-6 response element, −65/−52 IL-6RE, to which a 100-kDa protein complex is bound, has been identified on the JunB promoter. Leukemia inhibitory factor (LIF)-induced activation of JunB in M1 cells was also mediated via the −65/−52 IL-6RE. The STAT3 and CRE-like binding sites of the JunB promoter, identified as IL-6-responsive elements in HepG2 liver cells were found, however, to play no role in JunB inducibility by IL-6 in M1 myeloid cells. Conversely, the −65/−52 IL-6RE is shown not to be necessary for JunB inducibility by IL-6 or LIF in liver cells. It appears, therefore, that immediate early activation of JunB is regulated differently in M1 myeloid cells than in HepG2 liver cells. This indicates that distinct cis-acting control elements participate in cell type-specific induction of JunB by members of the IL-6 cytokine superfamily.

Toward dissecting the regulation of normal terminal differentiation and alterations in these regulatory processes that block differentiation, leading to leukemogenicity and its progression, our laboratory has isolated and characterized myeloid differentiation primary response (MyD) genes. MyD genes are activated in the absence of de novo protein synthesis in the autonomously proliferating murine M1 myeloid leukemia cells following induction for macrophage differentiation by lung conditioned medium, a potent physiological source for hematopoietic differentiation inducers, including interleukin-6 (IL-6)1 and leukemia inhibitory factor (LIF) (1–5). Sequence and expression analysis of MyD genes has led to the conclusion that the immediate early genetic response of myeloid cells to differentiation and growth inhibitory stimuli is complex. This complex response encompassed both known genes, including those for JunB, c-Jun, JunD, IRF-1, Egr-1, H3.3, H19, and ICAM-1, previously identified in the context of other biological systems, and novel genes, including those for MyD118, MyD116, and MyD88 (1–7). MyD genes also were shown to be induced in primary cultures of myeloid precursor-enriched bone marrow cells, with expression characteristics similar to what was observed in M1 cells (1–3). All the MyD transcription factors were stably induced following stimulation of M1 cells for differentiation by IL-6 or LIF, suggesting that they play a role in the initiation, progression and maintenance of the myeloid differentiation program (5).

The molecular nature of the mechanism by which IL-6 (or LIF) activates the immediate early expression of MyD genes upon induction of myeloid differentiation has not been elucidated. One approach to address this issue was to study the regulation of promoter elements of MyD genes that mediate the immediate early IL-6 response. Toward this end, JunB was chosen as a paradigm to investigate IL-6-mediated activation of MyD genes, because JunB is highly and stably induced by IL-6 and lacks any introns, which facilitated cloning of its promoter region. Here we report the identification and characterization of a novel cis-acting response element, which is essential for conferring IL-6 and LIF induction of JunB in M1 myeloid cells, but not in HepG2 hepatoma cells.

**MATERIALS AND METHODS**

**Cell Culture and Cytokine Treatments—**The murine myeloid leukemic cell line, M1, was grown in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Inc.) supplemented with 10% heat-inactivated horse serum (Life Technologies, Inc.) and 1% penicillin and streptomycin (P/S) (Life Technologies, Inc.). The cells were cultured in a humidified atmosphere with 10% CO2 at 37 °C. The human hepatoma HepG2 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown in DMEM supplemented with 10% heat inactivated fetal bovine serum (Life Technologies, Inc.) and 1% P/S at 37 °C in a humidified atmosphere with 5% CO2. For cytokine treatment the cells were exposed to either IL-6 (100 ng/ml) or a combination of IL-6 (100 ng/ml) plus IL-6 soluble receptor (IL-6SR) (50 ng/ml).

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1 The abbreviations used are: IL, interleukin; LIF, leukemia inhibitory factor; CAT, chlamylosphenol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; P/S, penicillin/streptomycin; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; Mut, mutant, WT, wild type; kb, kilobase(s); bp, base pair(s); EMSA, electrophoretic mobility shift assay; PKA, protein kinase A; JAK, Janus kinase; STAT, signal transducer and activator of transcription; GD, gross deletion; CAPS, 3′-cytidylylamino)propanesulfonic acid; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; SR, soluble receptor; RE, response element; CRE, cAMP response element; LS, linker scanning; TPA, 12-O-tetradecanoylphorbol-13-acetate.
Recombinant human IL-6 (rhuIL-6) was a generous gift from Amgen, Inc. (Thousand Oaks, CA). LIF and recombinant human IL-6SR were obtained from R&D Systems (Minneapolis, MN).

RNA Extraction, Northern Blotting, Hybridization, and DNA Probes—RNA was extracted from 1 × 10⁷ cells using Trizol (Life Technologies, Inc.). Total RNA (10 μg/lane) was electrophoresed on 1% agarose formaldehyde gels, and equal amounts of RNA in each lane were confirmed by equal intensity of ethidium bromide staining of ribosomal RNA bands and by probing with a β-actin probe. Northern blots, hybridization, and washing conditions were performed as described previously (8, 9).

 Probe for JunB (MyD21) was a cDNA cloned in our laboratory; the 1.5-kb insert was excised from pBluescript with EcoRI. The probe was agarose gel-purified and extracted with a Qiaquick gel extraction kit (Qiagen, Santa Clarita, CA). Human β-actin fragment was a commercially available probe (CLONTECH). DNA fragments for probes were labeled by random priming, using RadPrime DNA labeling kit (Life Technologies, Inc.) to a specific activity equal to or greater than 10⁹ cpm/μg.

Transient Transfections—M1 cells were seeded at a density of 5 × 10⁶ cells/10-ml plate 1 day prior to transfection, and 24 h later were transfected by the DEAE-dextran method, as described elsewhere (10). Briefly, 20 μg of total DNA was diluted in 0.75 ml of STBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄, 0.7 mM CaCl₂, and 0.5 mM MgCl₂) and added to 0.75 ml of 1 mg/ml DEAE-dextran (Sigma) solution. The 1.5-ml DNA/DEAE-dextran mix was then added to cells that were previously washed with STBS solution. The transfection was allowed to sit for 1 h at 37° C with agitation every 15 min. Afterwards, the cells were washed with 5 ml of STBS, 5 ml of DMEM, and 1% P/S, resuspended in 20 ml of DMEM, 10% horse serum, and 1% P/S, and split into two populations of 10 ml each. Cells were untreated or treated with IL-6 (100 ng/ml), or a combination of IL-6 (100 ng/ml) plus IL-6SR (50 ng/ml). For normalization of transfection efficiency, 2 μg of the pMLV-Luc plasmid, where the luciferase reporter gene is under the control of the constitutively active murine leukemia virus (MLV) promoter (11), was co-transfected with each construct. The transfected cells were harvested 20 h later, washed once with 5 ml of phosphate-buffered saline (Life Technologies, Inc.) and lysed with 120 μl of Reporter Lysis buffer (Promega, Madison, WI). 40 μl of cell extract was used for measuring luciferase activity, using a Lumat LB 9501 luminometer. Cell lysate volumes were adjusted, so

![Image](https://example.com/image1.png)

**Fig. 1. Identification of an IL-6-responsive region of the JunB promoter.** A, map of the P2 JunB promoter (~4.8 kb to +0.24 kb) from mouse Balb/c liver cells and cloning into pCAT-Basic vector. A genomic clone consisting of the region from ~4.8 kb to +242 bp of the gene for JunB was isolated and subcloned into the XhoI site of a pCAT-Basic vector. The mapped restriction sites and location relative to the JunB transcription start site are indicated. B, the JunB promoter (P2) region is IL-6-responsive. Reporter CAT activity in P2 and pCAT-Basic transiently transfected M1 cells, with and without IL-6 treatment (100 ng/ml). Cells were harvested 20 h later, and CAT assays were performed with lysates adjusted for equal luciferase activity. Acetylated products were separated on TLC gels and visualized by x-ray film. Relative CAT activity is defined relative to CAT expression in unstimulated P2 transfected M1 cells, which is set to 1.0. Results were quantified by PhosphorImager using Fuji Mac Bas version 2.0 software. Black boxes represent treatment with IL-6; white boxes are without IL-6. C, functional analysis of gross deletions of the JunB promoter in M1 cells. A series of 5'-nested gross deletions were constructed using P2. Construct names and sizes are indicated. Gray boxes represent the JunB promoter, while open boxes are the CAT coding region. All deletions contain +242-bp downstream region from the JunB transcription start site. M1 cells were transiently transfected with the gross deletion mutants, untreated or treated with IL-6 (100 ng/ml). Cells were harvested 20 h after transfection, and lysates adjusted for equal luciferase activity were measured for CAT activity. CAT induction is defined as the ratio of expression with IL-6 to without IL-6. Data are the average of three independent experiments, with standard deviations indicated.
that equal levels of luciferase activity were present in all samples. CAT activity was performed in 2 μl of [3H](chloramphenicol (NEN Life Science Products), 2 μl of 40 μM actetyl-CoA (Sigma), 18.8 μl of 2 μM Tris-HCl, pH 7.5, and adjusted to a total volume of 150 μl with 1X RNAse-free water. After the samples were incubated for 2 h at 37 °C, 2 μl of 40 μM actetyl-CoA was added, and further incubated for another 2 h. Samples were extracted twice with 200 μl of ethyl acetate (Fisher), lyophilized, and finally resuspended in 20 μl of ethyl acetate before spotting on a TLC silica gel plate (Baker-flex Silica Gel 1B, J.T. Baker). The acetylated products were separated following a 45-min run in 180 ml of 9:5 (v/v) chloroform:methanol solution and visualized by x-raying. Results were quantified by PhosphorImager using Fuji Mac Bas version 2.0 software.

Transfections into HepG2 cells were performed as described elsewhere (12), except cells were unstimulated or stimulated with IL-6 (100 ng/ml) for 24 h. Cell lysates were treated and assayed as described for M1 cells, except cells were lysed in 1 ml of Reporter Lysis Buffer and 4 μl of lysate was used for measuring luciferase activity.

Construction of Gross Deletions—To obtain the regulatory region of the JunB promoter, a genomic mouse Balc/c liver DNA library (CLONTECH) was probed with a random-primer JunB cDNA. A 4.8-kb upstream region and part of the gene for JunB up to +242 bp downstream of the transcription start site was isolated, mapped, and subcloned into the XbaI site of the pCAT-Basic vector (Promega) to obtain a construct, called P2, where the chloramphenicol acetyltransferase (CAT) reporter gene is under the control of the JunB promoter. A series of nested gross deletions (GD) were performed on the P2 construct by deleting the region upstream of the SpeI, XhoI, XcoI, EcoRV, XbaI, and PstI restriction sites to obtain the gross deletions SpeP2GD, XhoP2GD, XhoP2GD, EcoRV2P2GD, XbaP2GD, and PstP2GD, respectively.

XhoP2GD was used for constructing the linker scanning mutants, was obtained by subcloning the 1.5-kb regulatory region and part of the JunB transcribed region, up to +242 bp, into the HindIII site of pCAT-Basic, ensuring that the multiple cloning site of the pCAT-Basic vector was between the CAT reporter gene and the 1.5-kb upstream region of the JunB promoter.

The BfaGD was obtained by digesting XhoP2GD with HpaII and NcoI, and the resulting 1.3-kb fragment was isolated. This fragment was further digested with BfaI and XcmI, and the 355-bp fragment that was obtained was cloned into the XbaI site of pCAT-Basic.

The minimal promoter, −31GD, was obtained by using the PCR method, using the −5′-primer, −5′-TGAGAATATGCTGCTCGAGCAGG-3′ and the +3′-primer; −5′-CTGTCGACAGTATAAAAGCTTGG-3′, which changed a −242 bp of the JunB promoter and was amplified from 30 ng of double-plasmid DNA template, 0.5 mg bovine serum albumin, 1 mM dNTP, 5 mM primers, 1 μl Vent buffer (New England Biolabs, Beverly, MA), and 2.5 units of Taq DNA polymerase (New England Biolabs), heated at 94 °C for 1 min, annealed at 68 °C for 2 min, and subjected to 40 cycles using a Perkin-Elmer DNA Thermal Cycler. The primers used were selected with the aid of the program PCRPLAN of PCGENE 4.0. The PCR amplification was done with 25 ng of XhoP2GD DNA, 2.5 units of DNA polymerase (New England Biolabs), 3 mM dNTP, 5 mM Tris (pH 8.0), 2 mM MgCl2, 0.05% Nonidet P-40, 12% glycerol, 1 mg/ml bovine serum albumin, 0.5 mM dithiothreitol, and 1 mM AEBSF) at room temperature.

The primers used for sequencing using T7Sequenase version 2.0 kit (U. S. Biochemicals) in 0.25 ml of cold phosphate-buffered saline and lysed in RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 μM Tris (pH 8.0), 2 μg/ml aprotinin, 2 μg/ml benzamidine, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, and 100 μg/ml AEBSP). Cells were allowed to

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared from stimulated and unstimulated M1 and HepG2 cells, as described previously (13). Protein concentrations were determined according to the Bradford method using a Bio-Rad protein assay kit.

For cold oligonucleotide competition assays, 50- and 100-fold excess of unlabeled competitor oligonucleotides were mixed 5 min prior to the addition of labeled probes. The DNA probes (1 μg) were labeled by filling in 5′ cohesive ends with [α-32P]dATP (NEN Life Science Products) using 1 unit of Klenow enzyme (New England Biolabs) with 1 μg of double-stranded oligonucleotides. The products were separated on a 4% polyacrylamide gel (37:1 polyacrylamide:bisacrylamide, Roche Molecular Biochemicals) in 0.25% TBE (13). Binding reactions were set up in a 20-μl volume (unless otherwise indicated) by incubating probes (10,000 cpm) and 1 μg of poly(dI-dC)poly(dI-dC) (Amersham Pharmacia Biotech) with 5 μg of nuclear extracts in binding buffer (20 mM HEPES (pH 7.5), 70 mM KCl, 5 mM MgCl2, 0.05% Nonidet P-40, 12% glycerol, 1 mM bovine serum albumin, 0.5 mM dithiothreitol, and 1 mM AEBSP) at room temperature for 30 min. The complexes were electrophoresed on 5% nondenaturing polyacrylamide gel (37:1 polyacrylamide: bisacrylamide, Roche Molecular Biochemicals) in 0.25% TBE (1X TBE: 89 mM Tris borate, 89 mM boric acid, and 2 mM EDTA) buffer with 120 V at room temperature.

For cold oligonucleotide competition assays, 50- and 100-fold excess of unlabeled competitor oligonucleotides were mixed 5 min prior to the addition of labeled probes. The DNA probes (1 μg) were labeled by filling in 5′ cohesive ends with [α-32P]dATP (NEN Life Science Products) using 1 unit of Klenow enzyme (New England Biolabs) with 1 μg of double-stranded oligonucleotides. The products were separated on a 4% polyacrylamide gel (37:1 polyacrylamide: bisacrylamide, Roche Molecular Biochemicals) in 0.25% TBE (1X TBE: 89 mM Tris borate, 89 mM boric acid, and 2 mM EDTA) buffer with 120 V at room temperature.

Southwestern Blot Analysis—Whole extracts were prepared from 3 × 107 stimulated or unstimulated M1 cells, which were washed twice with 5 ml of cold phosphate-buffered saline and lysed in RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 μM Tris (pH 8.0), 2 μg/ml aprotinin, 2 μg/ml benzamidine, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, and 100 μg/ml AEBSP). Cells were allowed to
FIG. 3. A novel IL-6-responsive element is located between −65 and −52 of the JunB promoter. A, construction of linker scanning mutations. See “Materials and Methods” for details. LS, linker scanning mutants; EXOIII, exonuclease III digestion. B, linker scanning mutants were transiently transfected into M1 cells, untreated or treated with IL-6 (100 ng/ml). Location of the linker scanning mutations is based on their relative position from the transcription start site (+1). pCAT-SV40 contains a CAT reporter gene that is under the control of the SV-40 promoter.
Proteins bound to PVDF membrane were denatured twice with 6 M guanidine-HCl to without IL-6. Transfection and measurement of CAT activity with lysates adjusted for equal luciferase activity were performed as indicated under “Materials and Methods.” Three independent experiments yielded similar results. C, CAT induction. Induction is defined as the ratio of expression with IL-6 to without IL-6.

(a) Proteins bound to PVDF membrane were denatured twice with 6 M guanidine-HCl to without IL-6. Transfection and measurement of CAT activity with lysates adjusted for equal luciferase activity were performed as indicated under “Materials and Methods.” Three independent experiments yielded similar results. C, CAT induction. Induction is defined as the ratio of expression with IL-6 to without IL-6.

(b) Localization of IL-6 Response Elements on the JunB Promoter—We have shown that JunB is stably induced to high levels and that this induction is regulated at the transcriptional level (5). Therefore, JunB provided an excellent model to examine the IL-6-mediated regulation of MyD genes, by identifying and characterizing IL-6-responsive cis-regulatory elements of the JunB promoter. We set out to isolate a genomic clone of JunB that harbors the 5′-promoter region by screening a mouse genomic DNA library from BALB/c adult liver cells with a labeled murine JunB cDNA. A genomic clone, containing 4.8-kb upstream flanking sequences and 242 bp of the JunB transcribed region, was isolated. This promoter region was mapped and then subcloned into the XbaI site of the pCAT-Basic vector to obtain a construct, called P2, where the chloramphenicol acetyltransferase (CAT) reporter gene is under the control of the JunB promoter (Fig. 1A).

To evaluate the IL-6 responsiveness of the JunB promoter, P2 and pCAT-Basic were transiently transfected into M1 cells by the DEAE-dextran method (10), and the cells were either left untreated or treated with IL-6. The ability of IL-6 to induce CAT activity was examined (Fig. 1B). While expression of CAT was increased minimally in the presence of IL-6 in pCAT-Basic transfected cells, CAT was enhanced more than 3-fold by IL-6 in P2 transfected cells (Fig. 1B). This indicates that the 5′-upstream flanking sequences, up to 4.8 kb from the transcription start site of the JunB promoter, are sufficient for IL-6 responsiveness of JunB.

To identify cis-acting response elements in the 5′-flanking region of the mouse JunB gene that are IL-6-responsive, a series of 5′-nested gross deletions on P2 were constructed. Upstream regions were deleted to the SphI site of the pCAT-Basic vector to obtain a construct, called P2, where the chloramphenicol acetyltransferase (CAT) reporter gene is under the control of the JunB promoter (Fig. 1A).

and was used as a negative control for the IL-6 response (see schematic diagram of pCAT-SV40). Cells were transfected as indicated under “Materials and Methods,” and CAT assays were performed with lysates adjusted for equal luciferase activity. CAT induction is defined as the ratio of expression with IL-6 to without IL-6. Values are averages of three independent experiments and standard deviations (S.D.) are indicated. S.D. of linker scanning mutant −126/−113 and pCAT-SV40 are too small to be seen. Open bar represents the IL-6 inducibility of pCAT-SV40 transfected cells. Broken line indicates the IL-6 inducibility of XhoP2GD. A positive IL-6 response element, located between sequences −65 and −52 (arrow), was identified. C, locations of linker scanning mutations within the 194-bp upstream region of JunB are indicated in the schematic map. Position of mutations relative to previously identified regulatory elements (see Fig. 2) is indicated. D, the effect of IL-6 soluble receptor (IL6-SR) on IL-6 inducibility of the JunB promoter. The vectors XhoP2GD, −137/−124 LS mutant, and −65/−52 LS mutant were transfected into M1 cells, without or with IL-6 (100 ng/ml) alone, or in combination with IL-6SR (50 ng/ml). IL-6 and IL-6SR were added at the same time. Three independent experiments yielded similar results.

![Figure 4](image)

**Fig. 4.** The −65/−52 IL-6 response element is sufficient for IL-6 responsiveness of JunB. A, minimal promoter constructs. −31GD was used as a minimal JunB promoter (minimal promoter), since it does not respond to IL-6. Sequence between −75 and −42 of the JunB promoter was inserted upstream of −31GD in both the sense (JunBWT) or antisense orientation (rJunBWT). The −65/−52 IL-6RE, overlapping the CAAT motif and the IR sequence, is indicated by the open box. Sequence between −75 and −42, with mutations between −65 and −52 (crosses within CAAT and IR motifs), was also inserted upstream of −31GD in the sense orientation (JunBMut). Arrows indicate the direction of orientation and the box labeled Mutations represents the mutant sequences. The dark crosses within the arrows indicate that no JunB expression was observed. B, CAT activity. Minimal constructs were transfected into M1 cells, either untreated or treated with IL-6 (100 ng/ml), and CAT activity was determined. Transfection and measurement of CAT activity with lysates adjusted for equal luciferase activity were performed as indicated under “Materials and Methods.”
deletion promoter mutants were transiently transfected into M1 cells and assayed for CAT activity in the absence and presence of IL-6. Inducibility was calculated as the ratio of expression with IL-6 to that without IL-6. As shown in Fig. 1C, the smallest 5′-JunB promoter fragment generated, the 0.194-kb PstP2GD fragment, still maintained full IL-6 inducibility. It can be seen that P2 and PstP2GD conferred similar IL-6-induction of JunB. Therefore, we hypothesized that essential IL-6-responsive elements are contained within the 194-bp promoter region upstream of the JunB transcription start site.

Sequence analysis of the immediate 200-bp upstream region of the JunB promoter has revealed several potential regulatory elements that might be involved in IL-6 induction of JunB (Fig. 2). A TATA box is located 29 bp upstream of the transcription start site (14). An inverted repeat (IR), located between −57 and −50, was identified as a TPA- and PKA-inducible element (15). Other elements of potential importance are a CCAAT box, located −65 to −61, and a GC-rich area, located between −97 and −81. Furthermore, a CRE-like and a STAT3 binding site, which were originally identified as IL-6-inducible elements of the JunB promoter in the human hepatoma HepG2 cell line, are located between −149 and −124 (12).

Identification of a Novel IL-6 Response Element between −65 and −52 of the JunB Promoter—To identify the IL-6-responsive elements within the 194-bp upstream sequence of the JunB promoter, a linker scanning strategy was employed. A cluster of 13–14 bases was replaced, one at a time, with an unrelated sequence containing a SalI recognition site (Fig. 3A). This strategy, which maintained the size, and the topology, of the JunB promoter, as well as the spatial relationships between regulatory elements within the JunB promoter, minimized potential artifacts which can be generated by gross deletions. Since the overall topology of the promoter is not affected, the contribution of each individual response element can be better determined. XhoP2GD (−1.5 kb) was used as the backbone for the linker scanning mutants. In all, 15 linker scanning mutants were obtained that spanned the 194-bp upstream region (Fig. 3C).

The linker scanning (LS) mutants were transiently transfected into M1 cells, unstimulated or stimulated with IL-6. The pSV40-CAT vector, in which the CAT reporter gene is under control of the SV-40 promoter, was used as a negative control for IL-6 induction. The extracts were assayed for CAT activity, using lysate amounts that were normalized for luciferase activity. Of the 15 linker scanning mutants tested, only the −65/−52 LS mutant exhibited a severe reduction in IL-6 inducibility of JunB (Fig. 3B, see arrow). The marginal IL-6 induction that was observed for the −65/−52 LS mutant was comparable to the IL-6 induction of the pCAT-SV40 vector. This indicates that sequences between −65 and −52 contain a positive IL-6 response element that is necessary for induction of JunB by IL-6. This region was observed to contain a CCAAT box, located at −65 to −61 and the previously identified IR located at −57 to −50 (15). The IR element probably plays no role in the IL-6 response, since LS mutants −57/−48 and −55/−45, which overlap the entire IR region, still conferred full IL-6 inducibility (Fig. 3B). Also, mutating sequences between −137 and −124, which contains a CRE-like site (12) to which a 36-kDa protein binds (16), had no effect on the IL-6 responsiveness in M1 cells (Fig. 3B).

It was shown that the soluble form of the IL-6 receptor (IL-6SR) has an agonistic activity regarding IL-6 inducibility (17). Therefore, we wanted to examine what effect the addition of IL-6SR had on the IL-6 induction of CAT expression of the −65/−52 linker scanning mutant. Toward this end, XhoP2GD, −137/−124 LS mutant, and −65/−52 LS mutant were transfected into M1 cells, untreated or treated with IL-6 alone, or with a combination of IL-6 plus 50 ng/ml IL-6SR. The addition of IL-6SR to IL-6 increased the CAT activity of XhoP2GD and the −137/−124 LS mutant 4–5-fold relative to IL-6 treatment alone, whereas no effect was observed on the −65/−52 LS mutant (Fig. 3D). This result confirms the previous observations that the −65/−52 region contains a positive IL-6 response element. This is the first incident where this region, termed the −65/−52 IL-6 response element (IL-6RE), has been identified as a target for IL-6 signaling.

The Novel −65/−52 IL-6 Response Element Is Sufficient for IL-6 Inducibility—Mutating sequences between −65 and −52 within the 1.5-kb JunB promoter eliminated IL-6 induction, thereby allowing us to conclude that sequences within this region are necessary for IL-6 induction of JunB. We wanted to examine if the −65/−52 IL-6RE is sufficient for IL-6 inducibility. If this is the case, the −65/−52 IL-6RE should be sufficient to render the JunB minimal promoter responsive to IL-6. To determine this, we assayed the IL-6 responsiveness of minimal promoter constructs, where wild type (WT) and mutant (Mut)
sequences between −65 and −52 were inserted upstream of a JunB minimal promoter (Fig. 4A). WT sequences between −75 and −42 of the JunB promoter were cloned upstream of the JunB minimal promoter both in the sense and reverse orientation, called JunBWT and rJunBWT, respectively (Fig. 4A). The same element with mutations between −65 and −52, which were obtained from the linker scanning analysis, was also inserted upstream of the JunB minimal promoter; this minimal promoter construct was called JunBMut (Fig. 4A). WT sequences fully restored the IL-6 induction, while mutant sequences had no effect (Fig. 4, B and C). Furthermore, WT sequences in the reverse orientation still were able to maintain the IL-6 inducibility. Moving the element closer to the TATA box also did not have any effect on the IL-6 response. These data suggest that the element between −65 and −52 is not only sufficient for IL-6 responsiveness, but also has the characteristics of an IL-6-responsive enhancer element.

**Differential IL-6 Responsiveness of the −137/−124 and −65/−52 Linker Scanning Mutants in M1 Versus HepG2 Cells**—In HepG2 cells it was shown that elements between −149 and −124, called JRE-IL6, of the JunB promoter were necessary and sufficient for IL-6 responsiveness (12, 18). This effect was shown to be mediated by inducible binding of STAT3 (also known as APRF; Ref. 19) and a 36-kDa protein to these sequences (16). The results of these experiments suggested that sequences between −65 and −52 of the JunB promoter may play no role in the IL-6 response of JunB in liver cells (12). In contrast, in M1 myeloid cells we have shown that sequences between −137 to −124 of the JunB promoter are not important for IL-6 inducibility (see Fig. 3B). We hypothesized that the discrepancy between these two sets of observations may be due to cell type specificity.

When JunB expression was examined in HepG2 cells, it was observed that unlike in M1 cells, JunB was transiently induced following IL-6 treatment, with detectable levels of expression as early as 15 min, reaching peak levels at 30 min. At 2 h, JunB expression was barely detectable, increasing slightly at 3 h, and then declining to undetectable levels by 1 day (Fig. 5A). While JunB expression was superimposed with IL-6 in the presence of cycloheximide after 1 h, it was only slightly detectable with cycloheximide alone (Fig. 5A). Therefore, similar to what was observed with M1 cells, JunB is a primary response gene to IL-6 in HepG2 cells.

To test if cell type specificity of M1 and HepG2 cells plays a role in the regulation of the IL-6 response elements, wild type JunB promoter (PstP2GD) and LS mutants, −137/−124 and −65/−52 were transiently transfected into both M1 and HepG2 and assayed for IL-6 responsiveness. IL-6 inducibility was measured as CAT expression in IL-6-treated cells relative to CAT expression in untreated cells. As shown in Fig. 5B, a 3.2-fold induction was observed with PstP2GD in HepG2 cells, which was comparable to the 3.0-fold induction obtained in M1 cells. Overall, for the −137/−124 LS mutant, a 3.6-fold induction of CAT activity was observed in IL-6 stimulated M1 cells, while only a 1.6-fold induction was seen in IL-6 stimulated HepG2 cells. Conversely, for the −65/−52 LS mutant, only a 1.1-fold IL-6 induction was observed in M1 cells, as compared with a 3.1-fold IL-6 induction in HepG2 cells (Fig. 5B). These results further confirm that essential M1 and HepG2 IL-6 response elements of the JunB promoter reside within the 194-bp upstream regulatory region of JunB. Thus, sequences between −137 and −124 appear to be required for the IL-6 response in liver cells, whereas sequences between −65 and −52 appear to be required for the IL-6 response in myeloid cells.

The **STAT3 Binding Site Is Not Involved in the Regulation of JunB by IL-6 in M1 Myeloid Cells, but Is Critical in HepG2 Liver Cells**—In the previous section, it was shown that the CRE-like binding site, located between −137 and −124 of the JunB promoter, is not involved in the IL-6 response in M1 cells. We wanted to examine if the STAT3 binding site, located between sequences −147 and −138, is important for IL-6 inducibility in M1 cells. Toward this end, a deletion mutant was constructed, which eliminated the STAT3 and CRE-like binding sites; this construct was called BfaGD (Fig. 6A). The BfaGD deletion mutant contained the 113-bp upstream regulatory region and part of the JunB transcribed region up to +242 bp. This ensured that the STAT3 and CRE-like motifs, located between −149 and −124, were deleted but the −65/−52 IL-6RE, located between −65 and −52, was maintained (Fig. 6A).
The BfaGD mutant was transfected into both M1 and HepG2 cells and assayed for CAT activity in response to IL-6. Relative CAT activity was determined by comparing the expression of CAT in stimulated versus unstimulated cells. Similar to the −137/−124 LS mutant, the BfaGD deletion mutant in M1 cells could still confer IL-6 inducibility comparable to PstP2GD (Fig. 6B). In the presence of IL-6, CAT inducibility of BfaGD was 3.3-fold, which was similar to the inducibility observed with the control PstP2GD vector (3.9-fold). These results indicate that even in the absence of the −149 to −124 STAT3 and CRE-like binding sequences, more than a 3.0-fold IL-6 inducibility was maintained in M1 cells (Fig. 6B). In contrast, when BfaGD was transfected into HepG2 cells, CAT activity was only marginally increased 1.1-fold, compared with a relative increase of 4.2-fold for the PstP2GD (Fig. 6B). This shows that sequences between −149 and −124 are necessary for IL-6 responsiveness of JunB in HepG2 cells, which is in agreement with the published results (12, 18).

From these data it is clear that sequences between −149 and −124, which contain a STAT3 and CRE-like binding site, are not essential for IL-6 responsiveness of JunB in M1 myeloid cells, but are critical in HepG2 liver cells.

**LIF-mediated Induction of JunB Is Regulated via the −65/−52 IL-6RE—LIF**, although lacking structural homology to IL-6 (20, 21) and binding to distinct receptors (22), shares many biological properties with IL-6 (21, 23–25). Furthermore, we have shown that LIF and IL-6 trigger the same immediate early response in M1 cells (5). Therefore, we wanted to examine if the immediate early activation of JunB by LIF is mediated through the same −65/−52 IL-6RE in M1 versus HepG2 cells. Toward this end, M1 and HepG2 cells were transiently transfected with PstP2GD and the −65/−52 LS mutant, and assayed for CAT activity in the absence and presence of IL-6 and LIF. As shown in Fig. 7A, JunB induction by LIF, like by IL-6, was blunted in M1 cells transfected with the −65/−52 LS mutant. In contrast, induction of the JunB promoter by LIF appeared unaffected upon transfection of the −65/−52 LS mutant into HepG2 cells (Fig. 7B). Taken together these observations indicate that the −65/−52 IL-6RE in the JunB promoter participates in both IL-6 and LIF inducibility in M1 myeloid, but not in HepG2 liver cells.

**A 100-kDa Protein Complex Is Bound to the −65/−52 IL-6RE of the JunB Promoter**—The binding of nuclear factors to the −65/−52 IL-6 response element was examined by EMSA. Labeled JunBWT probe, alone (Fig. 8B) or in combination with excess unlabeled oligonucleotides (Fig. 8C), were mixed with nuclear extracts derived from M1 and HepG2 cells, untreated or treated with IL-6. When labeled JunBWT probe was used in gel shift experiments with nuclear extracts from unstimulated and IL-6-treated M1 cells, a single binding complex was detected, but no IL-6-induced binding was observed (Fig. 8B, top panel). Nuclear extracts from unstimulated M1 cells (Fig. 8B, lane 1) showed the same protein-DNA complex as extracts derived from IL-6-stimulated M1 cells (Fig. 8B, lanes 2–7). Addition of increasing amount of nonspecific double-stranded poly(dI-dC)poly(dI-dC) and protein extracts had no effect on the protein-DNA complex (data not shown). Using HepG2 extracts similar results were obtained (Fig. 8B, lower panel).

To determine the binding specificity of the protein-DNA complex, competition experiments were performed with 50- and 100-fold molar excess of unlabeled oligonucleotides in combination with labeled JunBWT probe. Using M1 and HepG2 cell extracts, excess unlabeled JunBWT oligonucleotides completely abolished the complex (Fig. 8C, lanes 2 and 3), while 50- and 100-fold molar excess of unlabeled JunBMut and STAT3/CRE oligonucleotides (Fig. 8C, lanes 4–7) did not compete it away. Furthermore, no binding of the complex was observed when labeled JunBMut probe was used in EMSA assays (data not shown). Taken together, these findings indicate that this nuclear protein complex is specifically bound to the −65/−52 IL-6RE.
IL-6/RE of the JunB promoter, and does not contain STAT3/CRE.

To determine the molecular weight of the protein complex that binds to the −65/−52 IL-6RE, we analyzed the proteins that bind to this element by Southwestern blotting. Whole cell extracts from IL-6-treated (10 min) M1 cells were electrophoresed on 7.5% SDS-PAGE gels and transferred to a PVDF membrane. Proteins on the membrane were first denatured with 6 M HCl-guanidine and then renatured by washing out the HCl-guanidine to approximately 0.1 M. The membrane was probed with labeled JunBWT probe (10,000 cpm/lane), and binding reactions were done at room temperature for 30 min. Protein-DNA complexes were resolved by electrophoresis on 5% nondenaturing polyacrylamide gel in 0.25 M Tris-HCl, pH 8.3, 0.1 M NaCl, 1 mM EDTA at 20°C. The positions of the protein-DNA complexes were determined by autoradiography and visualized with a Phosphor Imager. The 100-kDa protein complex is shown in Figure 8A.

The data presented provide evidence for a novel IL-6 response element, −65/−52 IL-6/RE, that is necessary and sufficient for the IL-6-induced activation of JunB in M1 myeloid cells. This element also was found to be required for JunB induction by LIF, another member of the IL-6 superfamily.
IL-6/LIF Induction of JunB

We have shown through EMSA and Southwestern analyses that a specific 100-kDa protein complex is constitutively bound to the −65/−52 IL-6RE in M1 cells before and after IL-6 stimulation. Since JunB is an immediate-early response gene, and, therefore, does not require de novo protein synthesis for induction of its mRNA, posttranslational processes would be expected to be involved in its regulation. Therefore, the complex that binds to the −65/−52 IL-6RE is probably in an inactive state, but following IL-6 stimulation is post-translationally modified, possibly through phosphorylation, to activate JunB transcription. Since the genetic mutagenesis analyses have revealed that this element does not play a role in HepG2 cells, it is likely that this factor, although present, is not post-translationally modified by IL-6 in these cells, and, therefore, is not responsible for JunB transcription. The IL-6/LIF signaling cascade, which is mediated via gp130, activates two distinct signaling pathways. One is the JAK-STAT pathway, which results in STAT3 activation. The other one is the activation of the RAS-mitogen-activated protein kinase pathway. In light of our findings, it is tempting to speculate that some kind of tissue specificity is intrinsic to these distinct signaling pathways. Accordingly, in HepG2 liver cells IL-6-induced JunB expression is mediated via the STAT3/STAT3-CRE complex of the JunB promoter via the JAK-STAT signaling cascade, whereas in M1 myeloid cells a different pathway is utilized, perhaps the RAS-mitogen-activated protein kinase pathway.

HepG2 Hepatoma Cells

M1 Myeloid Cells

**FIG. 9.** Schematic summary diagram depicting promoter elements that participate in IL-6-induced expression of JunB in M1 myeloid versus HepG2 liver cells. In M1 myeloid cells, the IL-6RE with the CAAT motif is important for the IL-6 mediated JunB expression. In contrast, in HepG2 liver cells, the STAT3 and CRE-like elements are crucial for the IL-6 response.

thereby extending our previous findings that LIF and IL-6 activate a common signaling cascade, leading to a similar immediate early MyD response upon induction of myeloid differentiation (5). Interestingly, we have found that the STAT3 and CRE-like binding sites of the JunB promoter, identified as IL-6 responsiveness elements in HepG2 liver cells (12), do not play a role in JunB inducibility by IL-6 in M1 myeloid cells. Conversely, the −65/−52 IL-6RE was shown not to be necessary for JunB induction by IL-6 in M1 cells. It appears, therefore, that immediate early activation of JunB is regulated differently in M1 myeloid cells than in HepG2 liver cells, suggesting that distinct cis-acting control elements are required for cell type-specific induction of JunB by members of the IL-6 superfamily (Fig. 9).

The −65/−52 IL-6RE contains two DNA motifs, a CAAT box located at the 5’ end of the −65/−52 IL-6RE, and the IR element located at the at the 3’ end of the −65/−52 IL-6RE (15). The IR element, located between sequences −57 and −50, was originally identified as an element that could confer phorbol ester and protein kinase A responsiveness in mouse hepatoma BW1 cells (15). It is unlikely that the IR element plays a role in the IL-6 inducibility of JunB in M1 cells because (i) linker scanning mutants −57/−48 and −55/−45, in which this entire region was mutated, did not show loss of IL-6 inducibility; and (ii) in M1 cells the IL-6 signaling cascade is distinct from TPA and PKA signaling pathways, and no JunB induction was observed following TPA and PKA treatment of M1 cells (5). Therefore, it seems likely that the CAAT box is important for IL-6 inducibility of JunB in M1 myeloid cells. A GC-rich area, located between sequences −97 and −81, which provides potential binding sites for several transcriptional activators such as Egr-1 and Sp-1, does not play a role in the IL-6 inducibility of JunB (data not shown). This suggests that these transcription factors are not involved in IL-6 responsiveness of JunB in M1 cells.

**FIG. 10.** Potential IL-6/LIF-RE-like motifs in the promoter regions of MyD genes. The consensus sequence was obtained from GCG sequence analysis software package (GCG, Madison, WI), using the Pretty command. *, inverse sequence. Bold bases indicate a match with the consensus sequence.

| Gene | Sequence | Position on promoter |
|------|----------|----------------------|
| JunB | GCGCAATGG | −67 to −58 |
| c-Jun | GCGCAATGGG | −102 to −93 |
| JunD | GCGCAATGGG | −74 to −65 |
| MyD118 | CACCAATGGG | Not Known |
| MyD88 | eCACCAATGGG | −614 to −605 |
| IRF-1 | GCGCAATGGG | −78 to −69 |
| H3.3 | TCACCAATGGG | −88 to −79 |
| Egr-1 | CCCCAATGG | −339 to −330 |
| | TCCCAATGG | −230 to −221 |
| Consensus | CGCGCAATGGG | N/A |

We have shown through EMSA and Southwestern analyses that a specific 100-kDa protein complex is constitutively bound to the −65/−52 IL-6RE in M1 cells before and after IL-6 stimulation. Since JunB is an immediate-early response gene, and, therefore, does not require de novo protein synthesis for induction of its mRNA, posttranslational processes would be expected to be involved in its regulation. Therefore, the complex that binds to the −65/−52 IL-6RE is probably in an inactive state, but following IL-6 stimulation is post-translationally modified, possibly through phosphorylation, to activate JunB transcription. Since the genetic mutagenesis analyses have revealed that this element does not play a role in HepG2 cells, it is likely that this factor, although present, is not post-translationally modified by IL-6 in these cells, and, therefore, is not responsible for JunB transcription. The IL-6/LIF signaling cascade, which is mediated via gp130, activates two distinct signaling pathways. One is the JAK-STAT pathway, which results in STAT3 activation. The other one is the activation of the RAS-mitogen-activated protein kinase pathway. In light of our findings, it is tempting to speculate that some kind of tissue specificity is intrinsic to these distinct signaling pathways. Accordingly, in HepG2 liver cells IL-6-induced JunB expression is mediated via the STAT3/STAT3-CRE complex of the JunB promoter via the JAK-STAT signaling cascade, whereas in M1 myeloid cells a different pathway is utilized, perhaps the RAS-mitogen-activated protein kinase pathway, which activates the 100-kDa/IL-6-RE complex of the JunB promoter. To test this interesting conjecture, characterization of the 100-kDa/IL-6RE complex and the signaling pathways which result in its activation is under way.

As shown in Fig. 10, many MyD genes, which are coordinately activated by IL-6/LIF, were also found to contain an IL-6/LIF-RE-like element, with a CCAAT motif, in their promoter regions. This suggests that the mechanism by which IL-6/LIF activates JunB expression, also operates to activate other MyD genes. Consistent with this notion, EMSA assays using synthetic oligonucleotides corresponding to IL-6/LIF response elements in the promoter regions of other MyD genes (Fig. 10) in conjunction with Southwestern analyses, have
shown that the 100-kDa protein complex is bound also to IL-6REs of other MyD genes.2

IL-6 and LIF are multifunctional cytokines that have been implicated in both healthy and diseased states (26, 27). In hematopoiesis, IL-6 and LIF are involved in M1 macrophage differentiation, maturation of megakaryocytes, and the enhancement of IL-3 dependent colony formation of primitive blast colony-forming cells (28). However, IL-6 and LIF also have been shown to be growth factors for myeloma and plasmacytoma cells (29). A defect in hematopoiesis in Fanconi anemia may be due to a deficiency in IL-6 production (30). On the other hand, IL-6 and LIF also play a major role in the synthesis of the acute phase proteins in response to injury and inflammation, such as the induction of fibrinogen, α1-antichymotrypsin, α1-acid glycoprotein, and haptoglobin, in the HepG2 and H35 hepatoma cell lines (23, 31). Thus, IL-6/LIF play a wide range of roles in the defense mechanisms, the immune response, hematopoiesis and acute phase reactions. In order for IL-6 and LIF to exert their multifunctional properties in different cell systems, they must activate distinct signaling pathways, leading to the regulation of different regulators, resulting in different IL-6/LIF responses. The data presented in this work provide first evidence for this notion. Extending these observations to other cell lines where JunB is an immediate early target of IL-6/LIF signaling, elucidating the signaling cascade involved, and the trans-acting factors they act on should aid in determining the scope of tissue versatility in IL-6/LIF signaling. Furthermore, these lines of investigation should provide an understanding of the molecular mechanisms by which IL-6/LIF produces both favorable and unfavorable effects on human health and may allow the development of new therapeutic strategies.

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