We previously noted that the initial receptor by which murine osteoclast precursors bind matrix is the integrin $\alpha_5\beta_3$ and that granulocyte-macrophage colony-stimulating factor (GM-CSF) decreases expression of this heterodimer by suppressing transcription of the $\beta_3$ gene. We herein report cloning of the $\beta_3$ integrin gene promoter and identification of a GM-CSF-responsive sequence. A 13-kilobase (kb) genomic fragment containing part of the $\beta_3$ gene was isolated by screening a mouse genomic library with a probe derived from the most 5'-end of a murine $\beta_3$ cDNA. A combination of primer extension and S1 nuclease studies identifies two transcriptional start sites, with the major one designated +1. A 1-kb subclone containing sequence $\sim$875 to +110 is transcriptionally active in a murine myeloid cell line. This 1-kb fragment contains consensus binding sequences for basal (Sp1), lineage-specific (PU.1), and regulated (signal transducer and activator of transcription) transcription factors. Reflecting our earlier findings, promoter activity is repressed in transfected myeloid cells treated with GM-CSF. Using deletion mutants, we localized a 109-base pair (bp) promoter region responsible for GM-CSF-inhibited $\beta_3$ transcription. We further identified a 19-bp sequence within the 109-bp region that binds GM-CSF-induced nuclear proteins by gel shift/competition assays. Mutation of the 19-bp sequence not only ablates its capacity to bind nuclear proteins from GM-CSF-treated cells, in vitro, but the same mutation, when introduced in the 1-kb promoter, abolishes its ability to respond to GM-CSF treatment. Northern analysis demonstrates that cycloheximide treatment abrogates the capacity of GM-CSF to decrease $\beta_3$ mRNA levels. In summary, we have identified a 19-bp cis-element mediating GM-CSF-induced down-regulation of $\beta_3$ by a mechanism requiring protein synthesis.

The osteoclast is a physiological polykaryon, related to, but distinct from, foreign body giant cells (1). Both multinucleated cells are derived by fusion of macrophages in a process apparently requiring attachment of the mononuclear precursors to matrix (2, 3). Cell-matrix interactions are mediated by several classes of receptors, including the family of heterodimers known as integrins (4–6). The integrin $\alpha_5\beta_3$ plays a critical role in osteoclastic bone resorption (7–10). In contrast, the integrin $\alpha_5\beta_2$, although closely related to $\alpha_5\beta_3$ in structure and sharing many of the same target ligands (4), is not expressed on mature bone-resorbing cells (11).

We have demonstrated that murine osteoclast precursors initially express and utilize the integrin $\alpha_5\beta_3$ and not $\alpha_5\beta_2$ for attachment to matrix (12). In this circumstance, $\alpha_5\beta_3$ and $\alpha_5\beta_2$ levels rise and fall, respectively, during formation of bone-resorbing polykaryons. The reciprocal changes in the two functional integrins are replicated by treating osteoclast precursors with GM-CSF, a cytokine that is important for osteoclastogenesis (13–15). Reflecting their surface heterodimers, $\beta_2$ and $\beta_3$ mRNAs fall and rise, respectively, in response to GM-CSF, whereas those of $\alpha_5$ are unaltered. GM-CSF-mediated inhibition of $\beta_3$ expression reflects decreased transcriptional activity of the $\beta_3$ gene (12).

In order to study the molecular mechanism by which integrin $\beta_3$ gene is regulated by the cytokine, we have isolated a $\beta_3$ integrin genomic fragment containing the transcriptional start sites and a portion of the regulatory domain of the gene. We demonstrate that the cloned sequence exhibits promoter activity and responds to GM-CSF in the same manner as the intact promoter in osteoclast precursors. Finally, we identify a 19-bp sequence within the $\beta_3$ promoter that mediates the GM-CSF responsiveness of this gene. This sequence does not contain consensus sequences for any transcription factor known to be regulated by GM-CSF and, as such, represents a novel GM-CSF response element.

**MATERIALS AND METHODS**

*Cloning of a Full-length Murine Integrin $\beta_3$ cDNA—Human $\beta_3$ integrin cDNA (provided by Dr. Eric Brown, Washington University School of Medicine) was labeled with digoxigenin to screen a 5'-stretch mouse kidney cDNA library (CLONTECH), and three clones were isolated and sequenced by dyeoxynucleotide chain termination (16). Because none contained a translational start site, the most 5' sequence available was used to screen a mouse brain cDNA library by the GeneTrapper Screening Method (Life Technologies, Inc.). Several new clones were isolated and completely sequenced.*

**Isolation of Murine Integrin $\beta_3$ Subunit Genomic Clones—**A mouse genomic library in FIX II vector (Stratagene) was screened with a 230-bp digoxigenin-labeled fragment derived from the most 5'-end of the longest cDNA clone. One positive clone, identified after examining 500,000 plaques, was purified by secondary and tertiary screenings. Phage DNA was prepared from the clone and was digested with NotI, resulting in two DNA fragments of 4 and 9 kb. Both fragments were...
subcloned into a pBluescript plasmid. Southern blot analysis showed that the 9-kb fragment positively reacted with the 230-bp probe derived from the most 5’-end of the longest cDNA clone.

** Primer Extension Analysis**—Primer extension was performed using total RNA prepared from mouse osteoclast precursors (bone marrow macrophages) and a 30-mer oligonucleotide complementary to a sequence 23 bp downstream of the 5’-end of the longest cDNA clone (Fig. 1). Excess oligonucleotide, end-labeled with [32P]ATP, was hybridized for 90 min at 65 °C with total RNA (previously denatured for 5 min at 95 °C). Annealed primer was extended with SuperScript II reverse transcriptase (Life Technologies, Inc.) at 42 °C for 60 min. The reaction products were treated with DNase-I (Life Technologies, Inc.) at 37 °C for 20 min, and the resulting mixture was separated on a denaturing gel, with an unrelated and previously defined marker to establish the size of specific bands.

**S1 Nuclease Analysis**—S1 nuclease experiments were performed using total RNA prepared from mouse bone marrow macrophages and a single-stranded 70-mer oligonucleotide comprising a sequence complementary to the first 44 bases of the 5’-end of the longest cDNA clone, the contiguous 16-base genomic sequence immediately upstream of the 5’-end of the clone, and a 10-base nonspecific sequence (as a control for nuclease activity; see Fig. 3B). The oligonucleotide, end-labeled with [32P]ATP, was mixed with excess total RNA, and the mixture was denatured at 75 °C for 10 min and then incubated overnight at 4°C with S1 nuclease (Boehringer Mannheim) at 37 °C for 60 min, and the resulting products were separated on a denaturing sequencing gel, using an unrelated and previously defined marker to establish the size of specific bands.

**Construction of a β3 Promoter-luciferase Reporter Plasmid and Deletion Mutants**—Sequence analysis indicates that there is a AclI site in the 5’-UTR of the full-length clone. A 30-mer oligo complementary to a sequence upstream of the AclI site was synthesized and end-labeled with digoxigenin. The 9-kb genomic fragment was digested with AclI, and the products were separated in an agarose gel, and hybridized with the 30-mer probe. A 1-kb fragment was obtained from the digestion reacted with the probe. Based on our screening strategy, this 1-kb fragment, which should contain the transcriptional start site, was subcloned into pGL3-basic plasmid containing the luciferase reporter gene, to construct a reporter plasmid named pGL3-1kb (+). A control reporter plasmid, pGL3-1kb (−), was also obtained by placing the 1-kb β3 promoter fragment in antisense orientation in front of the luciferase gene.

Deletion mutants of the 1-kb fragment were made by using the exonuclease III/fragmentation and nuclease P1 protocols. The 5’-end of two unique restriction sites in the multiple cloning sites region, one generating a 3’-overhang (KpnI) and another generating a 5’-overhang (MluI), I, made it possible to use this method. Following religation, the sizes of all mutants were determined by sequencing, using a common primer derived from vector sequence. The series of mutants generated in this manner is as follows: pGL3 (−796), with a sequence from −796 to +110; pGL3 (−627), from −623 to +110; pGL3 (−483), from −483 to +110; pGL3 (−340), from −340 to +110; pGL3 (−172), from −172 to +110; pGL3 (−63), from −63 to +110; and pGL3 (−28), from −28 to +110, and pGL3 (+10), from +10 to +110.

**Cell Culture and Transient Transfection**—FDC-P1/MAC1 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum (FBS) with 5% WEHI cell conditioned medium as a source of interleukin-3. Prior to transfection, cells were counted and spun down at 1400 × g for 7 min. The culture medium was then removed and saved, and the cells were resuspended in Opti-MEM (Life Technologies, Inc.) at a concentration of 12.5 × 106 cells/ml. 0.4 ml of this cell suspension was transiently cotransfected by electroporation at 325 V and 950 microfarads in Gene Pulser II (Bio-Rad) in a 0.4-cm Gene Pulser cuvette, with 20 μg of reporter plasmid and 0.5 μg of CMV-β-gal (GM-CSF (10 ng/ml) and 5 μg EDTA for 30 min on ice). Cells from two plates were scraped with rubber policeman, pooled, spun down, resuspended in 1.5 ml of cold PBS, and transferred to 2 ml microcentrifuge tubes. The cells were pelleted in a microcentrifuge for 30 s, media were removed and the cells were resuspended in 50 μl of hypotonic lysis buffer (10 mM Hepes-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 0.5 mM dithiothreitol, and phenylmethylsulfonyl fluoride were added freshly). Cells were lysed for 15 min on ice, at which time 32 μl of 10% Nonidet P-40 were added to the suspension, followed by vortexing of the tube for 15 s and incubating on ice for 10 min. Nuclei were spun down and resuspended in 100 μl of nuclease extraction buffer (20 mM Hepes-KOH, pH 7.9, 420 mM NaCl, 1.2 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 5 μg/ml pepstatin, and 5 μg/ml leupeptin). Dithiothreitol, phenylmethylsulfonyl fluoride, 4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin, and leupeptin were added freshly to the buffer. The extract was kept for 20 min on ice and spun down in a microcentrifuge. The supernatant (nuclear extract) was aliquoted, quickly frozen in a dry ice/ethanol bath, and stored at −70 °C.

**Site-directed Mutagenesis**—The point mutations were introduced in the context of the 1-kb promoter (pGL3-1kb (+)) by using a site-directed mutagenesis kit (Stratagene). Oligos used were 5′-CCCAACAGTTGCT-ACGTGTAaAGACCATCGGG-3′ and 5′-CCAGACGTGCTGTAaACACG-TaAaACCATGTTGGG-3′. Lowercase letters indicate the mutation sites. The oligos were purified by polyacrylamide gel electrophoresis. PCR was performed in a 50-μl volume with Pfu polymerase (Stratagene), 10 ng of pGL3-1kb (+) template, and 125 ng of each oligo under the following conditions: 1 cycle at 95 °C for 30 s; 15 cycles at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 12 min; and parking at 4 °C. The PCR was performed with Dpn I (1 units) for 60 min at 37 °C. XL1-Blue supercompetent cells were transformed with the Dpn I-treated PCR mixture as described in the instruction manual and plated on Ampicillin plates. Plasmids were prepared from individual colonies and sequenced to confirm the correctness of the introduced mutations.

**Northern Analysis**—Bone marrow macrophages were isolated and cultured in minimum Eagle’s medium containing 10% heat-inactivated FBS with daily supply of recombinant mouse macrophage colony-stimulating factor (10 ng/ml; R & D Systems; 48 h). Cells were transfected with 0.1 ng of CMV-β-gal plasmid (GM-CSF (10 ng/ml) and 5 μg EDTA for 6 h (PBS containing 0.1% BSA was used for the control). Total RNA was prepared from these cells using Trizol Reagent (Life Technologies, Inc.). For cycloheximide treatment experiments, macrophages were treated with GM-CSF (10 ng/ml) 30 min after the addition of cycloheximide (5 μg/ml). Cells then were cultured for 6 h before total RNA was isolated. 10 μg of each sample was separated on 1% agarose gel, and...
Northern analysis was performed using NorthernMax kit (Ambion, Austin, TX). A 1.4-kb mouse \(\beta_5\) cDNA fragment was labeled by BrightStar Psoralen-Biotin labeling kit (Ambion) and used as a probe in the Northern analysis (30 ng/ml). The signal was detected using BrightStar nonisotopic detection system (Ambion). The blot was stripped and replobed with 1.3-kb rat GAPDH probe (30 ng/ml, labeled in the same way.

**Fig. 1.** Nucleotide and amino acid sequence of a cDNA for mouse integrin \(\beta_5\) subunit (GenBank\textsuperscript{TM} accession number AF022110). A 3104-bp murine integrin \(\beta_5\) cDNA clone was completely sequenced. This sequence comprises a 294-bp 5' untranslated region (1–294), a 2367-bp coding region (295–2661), and a 443-bp 3' untranslated region (2662–3104). The translational start (ATG) and stop (TGA) codons are underlined. The deduced amino acid sequence is shown below the nucleotide data. The polyadenylation sequence is double underlined. Location of the primer (PE primer) used for the primer extension experiment (Fig. 2) is also shown below its complementary sequence (bp 24–54).
as the β probe) to normalize loading. To analyze the intensity of each band on the autoradiograms, the x-ray films were subjected to image analysis using ISS SepraScan 2001 (Integrated Separation Systems, Natick, MA). β mRNA levels were normalized to the GAPDH signal.

**RESULTS**

**Molecular Cloning of a Full-length Murine β5 cDNA—**Because integrin cDNA sequences are highly conserved across species (18, 19), we labeled a human β5 cDNA (20–22) and used it as a probe to screen more than 1,000,000 plaques derived from a 5'-stretch mouse kidney cDNA library (CLONTECH), which yielded three positive clones. Phage DNA was prepared, and the inserts were subcloned into pBluescript plasmid. Sequence analysis revealed that all three clones are incomplete, each terminating at +400, based on the human β5 sequence. Because we lacked sequence coding for the 5'-end of the cDNA, we used the GeneTrapper method (Life Technologies, Inc.) to rapidly screen a large number of clones. This approach, using as a probe the 5' sequence derived from our first round of cloning, resulted in isolation of several new clones, the longest containing 294 bp of the 5'-untranslated region, the entire coding sequence, and 443 bp of the 3'-untranslated region, including a polyadenylation sequence (Fig. 1) (sequence submitted to GenBank™; accession number AF022110). The deduced amino acid sequence is more than 90% identical to the human β5 protein (not shown).

**Isolation of Murine β5 Integrin Subunit Genomic Clones and Sequence Analysis of the 5'-Upstream Region—** Primer extension was performed to determine whether the largest β5 cDNA clone contains the complete 5'-untranslated region. The size of oligonucleotide used as primer (30 bp), plus the amount of untranslated sequence 5' of the ligation site of this primer (23 bp) would predict formation of a product of at least 53 bp (Fig. 1). A single band corresponding to a 59-mer oligonucleotide was detected (Fig. 2), indicating that the available β5 cDNA clone lacks 6 bases at its 5'-end. A 230-bp fragment derived from the most 5'-end of this cDNA was labeled and used to screen a mouse genomic library. After screening 500,000 plaques, one positive clone was identified, from which phage DNA was prepared. Restriction digestion analysis reveals the phage contains a 13-kb insert. A restriction map for the 13-kb was created by restriction digestion and Southern analysis (Fig. 3A).

![Fig. 2. Identification of the β5 cDNA 5'-UTR by primer extension analysis.](image)

![Fig. 3. Molecular cloning of a promoter for murine integrin β5 subunit gene.](image)
Detection of the phage DNA with Nofl yielded two fragments, 4 and 9 kb. Both fragments were subcloned into pBluescript plasmid. By Southern analysis, a probe containing the translational start site hybridized to the 9-kb clone. Sequencing of a portion of this 9-kb fragment, using primers (initially based on one vector arm and subsequently based on generated sequenc- es), provided approximately 1000 bases representing, in the 3’ to 5’ orientation, a 30-bp intron, 70 bp coding for the known 23-amino acid leader sequence, and a portion of the 5’-untranslated region. The combined results yielded a region subsequently identified as the proximal regulatory domain of the β₅ integrin gene (see Fig. 3B). Given the contiguous nature of the regulatory domain, 5’ untranslated region and leader se- quences, which end in the canonical AG sequence typical of intron/exon splice boundaries, this genomic fragment contains the first exon, which codes for the entire 5’-UTR and leader peptide, and the first base of the following codon. The size of the first intron, of which we have sequenced only 30 bp, is unknown.

Identification of the Transcriptional Start Site—To identify the 5’-end of the β₅ cDNA we turned to S1 nuclease protection assay (Fig. 4). Using this technique, two bands were detected, sug- gesting that this gene may have two transcriptional start sites, a documented feature of TATA-less promoters (23). The more prominent band, corresponding to a transcriptional start site, designated +1 (Fig. 3B). The discrepancy between this result and that obtained by primer extension (Fig. 2) probably reflects secondary structure near the GC-rich tran- scriptional start site inhibiting primer extension.

Sequence Analysis and Activity of Promoter Region—A 1-kb genomic fragment, derived from the original 9-kb genomic cDNA by restriction digestion and shown, by Southern analy- sis, to include the 5’-UTR of the β₅ cDNA, was subcloned into pGL3-basic, which contains the reporter gene luciferase. The resultant 1-kb plasmid, pGL3–1kb(+) and CMV-β-gal, with luciferase activity normalized to that of β-gal. The experiment was repeated three times, and a representative result is shown. Each bar is the mean of three replicates ± S.D.

**Figure 3.** The cloned 1-kb β₅ integrin promoter is transcriptionally active when transfected into murine myeloid progenitor cell line FDC-P1/MAC11 cells. Cells were transfected with the plasmids pGL3-SV40, pGL3-basic (promoterless), pGL3–1kb(–), pGL3–1kb(+), pGL3–726, pGL3–340, pGL3–172, pGL3–63, and pGL3–28, pGL3–(+10), and CMV-β-gal, with luciferase activity normalized to that of β-gal. The experiment was repeated three times, and a representative result is shown. Each bar is the mean of three replicates ± S.D.
Cloning and Regulation of Murine β5 Integrin Promoter

Fig. 6. The 1000-bp proximal β5 promoter confers GM-CSF-mediated repression of integrin β5 gene expression. FDC-P1/ MAC11 cells were transfected with pGL3-promoter (SV40), pGL3–1kb(+), or CMV β-gal, with luciferase activity normalized to that for β-gal. The experiment was repeated three times, and a representative result is shown. Each bar is the mean of three replicates ± S.D.

Fig. 7. Localization of a 109-bp region mediating GM-CSF-dependent repression of the integrin β5 gene transcription. FDC-P1/MAC11 cells were cultured, transfected with a series of deletion constructs of the β5 promoter, and assayed as described in Fig. 6. The experiment was independently repeated three times, and a representative result is shown. Each bar is the mean of three replicates ± 5D.

Fig. 8. Identification of 34-bp sequence within the 109-bp region binding GM-CSF-induced nuclear proteins. A, schematic locations of the four overlapping oligos (I, II, III, and IV) spanning the 109-bp region. B, EMSA with oligos I, II, III, and IV and nuclear extracts from GM-CSF-treated (48 h) or untreated BMMs. Nuclear extracts were incubated with 1 × 10^6 cpm of each oligo labeled with 32P, separated by polyacrylamide gel electrophoresis, dried, and autoradiographed.

EMSA studies is indeed functional in mediating GM-CSF inhibition of β5 gene transcription, we first generated a set of point mutations (Fig. 10A) in oligo III that abolished its capacity to compete in EMSA for binding of the nuclear proteins derived from GM-CSF-treated or untreated BMMs. The mutant oligo III (Fig. 10B, M) failed to compete for binding of the nuclear proteins in bands A, B, and C (lanes 4, 5, 9, and 10), whereas wild-type oligo III (III) did so efficiently (lanes 2, 3, 7, and 8). These findings show that the 19-bp sequence containing the indicated point mutations did not bind the nuclear proteins...
in bands A, B, and C. We then introduced the same point mutations in context of the 1kb promoter (pGL3–1kb(1)) and named the resulting plasmid mpGL3–1kb(1). When mpGL3–1kb(1) was transfected into FDC-P1/MAC11 cells, its activity was no longer inhibited by GM-CSF treatment, whereas the wild-type reporter continued to respond (Fig. 11). Thus, we have identified a cis-element in the β5 promoter that mediates down-regulation of transcription of this gene by GM-CSF.

FIG. 9. Identification of a 19-bp sequence within the 34-bp oligo III as sufficient for binding the GM-CSF-induced nuclear proteins. A, deletion mutants based on oligo III (a–g). B, EMSA/competition assays with the deletion mutants shown in A. A 20× or 100× excess of each unlabeled mutant oligo was premixed with 1 × 10^5 cpm of labeled oligo III, prior to EMSA, performed as described in Fig. 8B. C, EMSA/competition assays, as performed in B, but using nuclear extracts from GM-CSF-treated cells.

FIG. 10. Point mutations in the 19-bp β5 integrin promoter sequence abolish its ability to bind nuclear proteins from both GM-CSF-treated and untreated BMMs. A, identity of the four point mutations introduced into oligo III. The 19-bp sequence identified in Fig. 9 is boxed; the mutated nucleotides are in lowercase letters, with their wild-type counterparts above or below. B, EMSA/competition assays were repeated as described in Fig. 9, B and C, with wild-type oligo III (III) or the point mutated oligo III (M).

FIG. 11. Mutation of the 19-bp sequence in the 1-kb β5 promoter abrogates its capacity to mediate GM-CSF responsiveness. The same four point mutations described in Fig. 10A were introduced in the context of the 1-kb β5 promoter, generating the construct mpGL3–1kb(1). GM-CSF-treated or untreated FDC-P1/MAC11 cells were transfected with wild-type promoter (pGL3–1kb(1)) or the mutant form, mpGL3–1kb(1), with CMV-β-gal plasmid as control and assayed as described in Fig. 6. These studies were performed four times, with a representative result shown. Each bar is the mean of three replicates ± SD.
GM-CSF, in other circumstances, invariably stimulates gene expression, our observations represent a novel role for the cytokine, namely transcriptional inhibition.

The coding region of the human integrin β₅ gene contains 14 exons within 46 kb of genomic DNA (28). The 5’-UTR resides in a separate exon, separated by an uncloned intron (29). Because the β₅ and β₃ integrin cDNAs are highly homologous (20), the β₃ integrin gene, like that of β₅ (28), may bear a large first intron. If this is so, it would be difficult to isolate a genomically cloned containing the promoter region of the murine β₅ integrin gene without identifying the 5’-untranslated end of the cDNA. With this in mind, our first exercise was to clone a full-length β₅ murine cDNA. Using the most 5’-untranslated sequence as a probe, we screened a mouse genomic library and isolated a 13-kb fragment containing part of the coding region. A combination of primer extension and S1 nuclease analysis identified two potential transcriptional start sites. Similar to other integrin promoters (30–33), the β₅ gene does not contain a classical TATA element (25) in the vicinity of the transcriptional start site. The first kb of the β₅ promoter, in contrast, accommodates four consensus sites for the B and myeloid cell-specific transcription factor, Pu.1, which plays an essential role in osteoclastogenesis (34). Most importantly, the 1-kb fragment bearing the putative transcriptional start site contains a functional promoter as it enhances transcription at least 15-fold in transfected cells. Deletion analysis of the initial 1-kb promoter shows that basal promoter activity resides between –28 and +10, a region that contains a consensus recognition site for Sp1. Because Sp1 can act as an initiator of transcription in genes lacking a TATA box (35), the finding of a consensus Sp1 binding site suggests that it may function in this role in the β₅ promoter. Most importantly, by a combination of transient transfection studies and EMSAs, using both deletion and mutation of the regulatory region of the integrin β₅ gene, we have identified a potentially novel 19-bp sequence responsible for GM-CSF-dependent transcriptional inhibition.

We were concerned because our EMSA experiments had been performed using BMMs as a source of protein, whereas our transfections used exclusively the FDC-P1 cell line. To address this issue, we repeated the EMSA studies using oligos I–IV and nuclear proteins from FDC-P1 cells treated with either GM-CSF or PBS-containing medium as control. In all instances, the pattern of bands was identical to that obtained using BMMs (data not shown), results that buttress the approach of using primary cells and transformed lines to dissect the mechanism of GM-CSF regulation of the β₅ gene.

We initially attempted to determine whether the 19-bp GM-CSF-responsive element in the β₅ promoter can mediate GM-CSF-dependent inhibitory effects on heterologous promoters, subcloning three tandem repeats of the 19-bp sequence upstream of the thymidine kinase minimal promoter. However, when this reporter plasmid was transfected into FDC-P1/MAC11 cells not treated with GM-CSF, only background luciferase activity was detected (data not shown). When the same three copies of the 19 bp sequence were subcloned in front of the SV40 promoter (pGL3-promoter from Promega), high promoter activity was detected in transfected FDC-P1/MAC11 cells. However, GM-CSF treatment does not decrease the activity of this construct (data not shown). SV40 promoter is a strong TATA-box based viral promoter, whereas integrin β₅ promoter is TATA-less. Such differences may contribute to the failure of the 19-bp sequence to mediate the inhibitory effect of the cytokine on the SV40 promoter. Thus, it is likely that the characterized GM-CSF-responsive sequence functions in a promoter-specific manner.

GM-CSF is a member of a cytokine superfamily that utilizes...
a number of major pathways, including those involving c-Myc (36), STAT5 (36), IRS-2 (37), and Ras (36), to transduce receptor-mediated signaling from the cell surface to the nucleus. STAT proteins are transcription factors that, in their inactive form, reside in the cytoplasm (38–40). Binding of a wide range of cytokines to their cognate plasma membrane receptor activate members of the Janus kinase family, the major substrates of which are STAT proteins. Phosphorylated STATs dimerize and translocate to the nucleus, where they almost always activate transcription (40). STAT5, which exists in both mice and humans as two isoforms, STAT5A and STAT5B (41, 42), in other circumstances mediates GM-CSF-dependent transcription (40). Although the 19-bp sequence contains two STAT-like sequences, they do not reside in the GM-CSF-responsive sequence and thus play no role in the mechanism by which the cytokine modulates expression of the gene. To show that the nuclear proteins binding to the 19-bp sequence are indeed STAT proteins, we performed EMSA experiments with anti-STAT5A and anti-STAT5B antibodies and found, as expected, that these reagents fail to supershift the bands (data not shown). Thus, we conclude we have identified a GM-CSF response element binding transcription factor(s) other than STATs. The fact that this response element does not bind proteins known to be involved in transcriptional control by GM-CSF suggests that we may have identified a novel response element.

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