MOLECULAR AND CELLULAR BIOLOGY, May 1990, p. 2002–2011
0270-7306/90/052002-10$02.00/0

Vol. 10, No. 5

Regulatory Elements Responsible for Inducible Expression of the Granulocyte Colony-Stimulating Factor Gene in Macrophages

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Received 30 October 1989/Accepted 10 January 1990

Granulocyte colony-stimulating factor (G-CSF) plays an essential role in granulopoiesis during bacterial infection. Macrophages produce G-CSF in response to bacterial endotoxins such as lipopolysaccharide (LPS). To elucidate the mechanism of the induction of G-CSF gene in macrophages or macrophage-monocytes, we have examined regulatory *cis* elements in the promoter of mouse G-CSF gene. Analyses of linker-scanning and internal deletion mutants of the G-CSF promoter by the chloramphenicol acetyltransferase assay have indicated that at least three regulatory elements are indispensable for the LPS-induced expression of the G-CSF gene in macrophages. When one of the three elements was reiterated and placed upstream of the TATA box of the G-CSF promoter, it mediated inducibility as a tissue-specific and orientation-independent enhancer. Although this element contains a conserved NF-κB-like binding site, the gel retardation assay and DNA footprint analysis with nuclear extracts from macrophage cell lines demonstrated that nuclear proteins bind to the DNA sequence downstream of the NF-κB-like element, but not to the conserved element itself. The DNA sequence of the binding site was found to have some similarities to the LPS-responsive element which was recently identified in the promoter of the mouse class II major histocompatibility gene.

Various genes are expressed at high levels exclusively in a specialized cell types. By introducing modified cloned genes into living cells, many regulatory sequences which specify the appropriate gene expression have been defined (36). Since hematopoietic cells express a set of genes in a tissue-specific manner, the mechanisms of gene expression in B and T lymphocytes were extensively studied as a model system for cell-type-specific gene expression. Typical elements identified in immunoglobulin genes are the octamer sequence (ATTTGCAT), located 60 to 80 base pairs (bp) upstream of the transcription initiation site of immunoglobulin heavy- and light-chain genes, and the NF-κB element (GGGGGATTTCC), situated in the enhancer region of the immunoglobulin κ chain gene (2). The NF-κB-like element was also found in the promoter regions of the interleukin-2 (IL-2) gene, the IL-2 α-receptor gene, and human immunodeficiency virus (11, 15) and suggested to be involved in the activation of these genes in T cells. In contrast to B and T lymphocytes, few studies have been reported on gene regulation in macrophages or monocytes which produce several monokines (23).

Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein which regulates proliferation, differentiation, and maturation of the progenitor cells of neutrophilic granulocytes (6, 17, 21). Marked granulopoiesis during inflammatory processes is mediated mainly by G-CSF released into the serum (3). Peritoneal macrophages produce G-CSF in response to bacterial endotoxins such as lipopolysaccharide (LPS) (7, 18, 24, 34), whereas IL-1 or tumor necrosis factor α stimulates endothelial cells and fibroblast cells to produce G-CSF (13, 28). In addition, some carcinoma cells constitutively secrete G-CSF in large quantities (33). A G-CSF chromosomal gene of approximately 2.5 kilobases located on human chromosome 17 or murine chromosome 11 consists of four exons, and the promoter region of human and mouse G-CSF chromosomal genes is highly homologous up to 300 bp from the transcription initiation site (22, 32). In this region, a typical octamer sequence (ATTTGCAT) is located at −110 bp upstream of the TATA box. Furthermore, an NF-κB-like sequence (GAGATTCCCC) was found at −180 in the promoter of the mouse G-CSF gene, although it deviates slightly from the consensus sequence [GG(N)$_6$CC] for the NF-κB element proposed by Clark and Hay (5). Since a nearly identical NF-κB-like sequence was found in the promoter of not only G-CSF but also granulocyte-macrophage CSF (GM-CSF) and IL-3 genes, the element was referred to as a CSF box (32) or as conserved lymphokine element 1 (19).

Previously we prepared a nested series of mutants with linker-scanning mutations and internal-deletion mutations in the promoter of the mouse G-CSF chromosomal gene, joined to the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene (M. Nishizawa, M. Tsuchiya, R. Watanabe-Fukunaga, and S. Nagata, J. Biol. Chem., in press). By introducing these hybrid genes into human carcinoma cells producing G-CSF constitutively, it was shown that at least three elements (G-CSF promoter elements 1 through 3 [GPE1 through GPE3]) are required for the constitutive expression of the G-CSF gene in carcinoma cells. GPE1 consists of the CSF box and a 23-bp sequence downstream of the CSF box, and GPE2 is an octamer sequence.

In this study, we found several macrophage and monocyte-macrophage cell lines which could be stimulated by LPS to produce G-CSF. In these macrophage cell lines, GPE1 through GPE3 were essential for the inducible expression of the G-CSF gene. To elucidate the contribution of each element to G-CSF gene expression, the reiterated GPE was placed upstream of the TATA box of the G-CSF promoter and introduced into macrophage cell lines. These studies have indicated that multiple copies of GPE1 can function as an LPS-inducible regulatory element in macrophages. Furthermore, by using footprint analysis and the gel retardation assay, we showed that nuclear factors prepared from macrophage cell lines can bind to the proximal part of GPE1, but not to the NF-κB-like CSF box itself.

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MATERIALS AND METHODS

Cells. The simian virus 40-transformed mouse macrophage cell line BAM3 (25) was maintained in Dulbecco modified Eagle medium (DMEM; Nissui) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Inc.). The mouse monocyte-macrophage cell line PU5-1.8 (ATCC TIB61), the mouse macrophage cell line IC-21 (ATCC TIB186), the human lymphoblastoid cell line Namalwa (ATCC CRL 1432), and the human lymphoma cell line Jurkat (8) were maintained in RPMI 1640 medium (Nissui) containing 10% FCS. Human epithelioid carcinoma HeLa cells (ATCC CCL2) were grown in minimal essential medium supplemented with 10% FCS.

Assay for G-CSF activity. G-CSF activity was assayed by stimulation of [^{3}H]thymidine incorporation into the factor-dependent mouse NFS-60 myeloid leukemia cells as described previously (31). One unit of activity represents the concentration of G-CSF which gives half-maximal stimulation with 5×10^4 cells per 100 μl.

Northern (RNA) hybridization. Mouse BAM3 cells (2×10^6) were seeded onto 10-cm plates and grown in DMEM containing 10% FCS. When the cells became confluent, they were incubated in medium with or without 10 μg of LPS (*E. coli* O111:B4; Difco Laboratories) per ml. At the indicated time, they were washed with phosphate-buffered saline, and total RNA was prepared by the guanidine thiocyanate-CsCl method of Chirgwin et al. (4). RNA (5 μg) was denatured by heating at 60°C for 5 min in 2.2 M formaldehyde–50% deionized formamide and electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde (16). The fractionated RNA was transferred to a nitrocellulose filter and hybridized with the ^{32}P-labeled *Eco*RI fragment of pMG2 (31).

Transfection and CAT assay. Transfections of plasmid DNA into BAM3 cells and HeLa cells were carried out by the calcium phosphate coprecipitation method or the DEAE-dextran method as described previously (9). After a glycerol shock for 1 min, cells were incubated for 12 h in DMEM containing 10% FCS. The cells were then exposed to the medium with or without 10 μg of LPS per ml for 48 hrs and harvested for the CAT assay.

Suspension cultures of PU5-1.8, Jurkat, and Namalwa cells were transfected by the DEAE-dextran method as described previously (8). In brief, 5×10^6 to 6×10^6 cells were washed twice with TBS (25 mM Tris hydrochloride [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$), suspended in 1 ml of TBS containing 500 μg of DEAE-dextran (Pharmacia, Inc.) per ml and 10 μg of plasmid DNA, and incubated for 30 min at room temperature. After incubation, PU5-1.8 cells (but not Jurkat cells) were treated for 60 min at 37°C with 0.1 mM chloroquine in RPMI medium containing 10% FCS and cultured in RPMI medium for 12 h. For Namalwa cells, 0.1 mM chloroquine was included in the mixture of DEAE-dextran and DNA, and the cells were incubated for 90 min at room temperature. After transfection, the cells were exposed for 48 h to fresh medium containing 10 μg of LPS per ml or for 36 h to medium containing 25 μg of concanavalin A (Con A) per ml. The CAT assay was carried out as described previously (9) with 30 to 200 μg of extract proteins. [^{14}C]chloramphenicol was incubated with the extract for 2 to 5 h at 37°C, and the products were separated by thin-layer chromatography.

Plasmid construction. Construction of plasmids carrying linker-scanning mutations and internal-deletion mutations in the promoter of G-CSF gene has been described previously (Nishizawa et al., in press). To construct plasmids containing repeated GPEs in their promoters, the oligonucleotides shown in Fig. 1 were chemically synthesized by the phosphoramidite method with a DNA synthesizer (model 381A; Applied Biosystems, Inc.).

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          -193                                              -152
GPE1 :  CTAGAGAGATTCCCCGATTTCACAAAAACTTTCGCAAACAGCTTTT
            TCTCTAAGGGGCTAAAGTGTTTTTGAAAGCGTTTGTCGAAAACTAG

          -198              -181
GPE1d:  TCGAGGAACAGAGATTCCCCGAG
            CCTTGTCTCTAAGGGGCTCAGCT

          -182                                  -152
GPE1p:  TCGAGATTTCACAAAAACTTTCGCAAACAGCTTTTG
            CTAAAGTGTTTTTGAAAGCGTTTGTCGAAAACAGCT

          -122                  -103
GPE2 :  GATCCATCAAATTTGCATAAATCCA
            GTAGTTTAAACGTATTTAGGTCTAG

            -104
GPE3 :  TCGACCCCTGGGAAGTTATTACTAAGCCTGAGTTGCCCCA
            GGGGACCCTTCAATAATGATTCGGACTCAACGGGGT

                        -51
        GCCCCAGGTAATTTCCTCCC
        CGGGGTCCATTAAAGGAGGG

          -148                                  -119
GPE4 :  TCGAGCAACCCCCTGCATTGTCCTGAGCTATCATCG
            CGTTGGGGGACGTAACAGGACTCGATAGTAGCAGCT
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FIG. 1. Oligonucleotides synthesized for construction of plasmids containing GPEs.

As a vector, pUC19 was modified by inserting an *Xho*I linker at the *Sac*I site and designated as pUC-SX. The oligonucleotides, GPE1d, GPE1p, and GPE4 were inserted at the *Sal*I-*Xho*I site of pUC-SX (pGPE1d-1, pGPE1p-1, and pGPE4-1), and the GPE1 oligonucleotide was incorporated between the *Xba*I and *Bam*HI site of pUC-SX (pGPE1-1). To generate pGPE2-1 and pGPE3-1, *Bam*HI-digested pUC-SX or *Sal*I- and *Sma*I-digested pUC-SX were used as vectors, respectively. Multimerization of the regulatory sequence was performed essentially as described previously (35). In brief, pGPE1-1 was digested with *Sca*I and *Sal*I or with *Sca*I and *Xho*I, and the DNA fragment containing GPE1 was ligated at *Sca*I- and *Sal*I-*Xho*I-compatible sites to produce pGPE1-2 containing two tandem GPE1s. pGPE1-2 was further digested with *Sca*I and *Sal*I or with *Sca*I and *Xho*I, and pGPE1-4 containing four tandem GPE1 elements was constructed by a similar method. Plasmid DNAs containing tetramers of other regulatory elements, GPE1p, GPE1d, GPE2, GPE3, and GPE4, were prepared by the same procedure, starting from the corresponding plasmid containing the monomer of each regulatory sequence.

One of the 5′ deletion mutants (pBP-NC13) which contains the promoter sequence up to −67 from the transcription initiation site was used for construction of a series of plasmids (pTA-E14 through pTA-E44) containing the tetramer of the regulatory element upstream of the TATA box. The polylinker region of pSV0B cat (Nishizawa et al., submitted) was transferred to pBP-NC13, and the *Sac*I site in the polylinker was changed to an *Xho*I site by using the *Xho*I linker to produce pBPA-TATA. Plasmids pGPE1-4, pGPE1d-4, pGPE1p-4, pGPE2-4, pGPE3-4, and pGPE4-4 were digested with *Sal*I and *Xho*I. *Sal*I-*Xho*I DNA fragments containing the multimerized regulatory sequence were inserted between the *Sal*I and *Xho*I sites of pBPA-TATA in both the correct and reverse orientations. The structures of the resultant constructions were confirmed by DNA sequence analysis.

Gel retardation assay. Nuclear extracts were prepared by

the method of Shapiro et al. (29) from BAM3 cells or PU5-1.8 cells. As a probe, pGPE1-1 was cleaved with *Hin*dIII, and its 3′ end was labeled with [α-^{32}P]dCTP (specific activity, 6,000 Ci/mmol) and the Klenow fragment of *E. coli* DNA polymerase I. The DNA was digested with *Xho*I, and a ^{32}P-labeled 91-bp *Hin*dIII-*Xho*I DNA fragment (ca. 5 × 10^6 Cerenkov counts/pmol) was isolated by electrophoresis on 0.7% low-melting-point agarose gel. For the binding reaction, 2 μg of nuclear extract was preincubated for 15 to 30 min on ice in a final volume of 14 μl containing 20 mM Tris hydrochloride (pH 8.0), 100 mM KCl, 2% (wt/vol) polyvinyl alcohol (Sigma Chemical Co.), 10% (vol/vol) glycerol, and 1 μg of poly(dI-dC) (Pharmacia). After the addition of 1 μl of the labeled probe (2 fmol), the reaction mixture was incubated for 30 min at 30°C and analyzed by electrophoresis on a 4% polyacrylamide gel in buffer containing 50 mM Tris, 380 mM glycine, 2 mM EDTA (pH 8.5), and 2.5% (vol/vol) glycerol. As specific and nonspecific competitors, *Hin*dIII-*Eco*RI DNA fragments were prepared from plasmids containing the tetramer of each GPE element (pGPEn-4) and were included in the preincubation mixture.

Footprint analysis. DNase I footprint analysis was performed essentially as described by Jones et al. (12). DNA-binding reactions were carried out in a 30-μl volume with 5 fmol of labeled DNA fragment, 1 μg of poly(dI-dC), and 20 μg of nuclear extract as described above. After incubation at 30°C for 30 min, the reaction mixture was diluted with an equal volume of solution containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$ and incubated for 60 s at room temperature with 3 μl of DNase I (10 μg/ml; Worthington Diagnostics). The reactions were terminated by the addition of 60 μl of a mixture containing 20 mM EDTA, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1% sodium dodecyl sulfate, 50 μg of tRNA per ml, and 100 μg of proteinase K per ml. The mixtures were incubated at 37°C for 15 min and extracted with phenol-chloroform. DNAs were recovered by ethanol precipitation and electrophoresed on an 8% polyacrylamide–urea sequencing gel.

A neocarzinostatin protection assay was carried out by the method of Koepsel et al. (14). In brief, after the binding reactions of DNA with nuclear proteins, the mixtures were adjusted to 2 mM dithiothreitol and 1 mM EDTA. Neocarzinostatin (1 U; Yamanouchi Co.) was added to the mixtures, which were incubated for 10 or 30 min at room temperature. The products were analyzed as described for the DNase I footprint analysis.

RESULTS

Induction of G-CSF in mouse macrophage and monocyte-macrophage cell lines. Mouse peritoneal macrophages or human monocytes produce G-CSF in response to endotoxin stimulation (7, 18, 24, 34). To study the induction mechanism of the G-CSF gene in macrophages or monocytes, we examined several macrophage and macrophage-monocyte cell lines for the expression of G-CSF. BAM3, PU5-1.8, and IC-21 cells were incubated for 24 h with 10 μg of LPS per ml or 25 μg of ConA per ml, and G-CSF activity in the medium was determined by measurement of its ability to stimulate proliferation of mouse NFS-60 cells (31). G-CSF activity (300 to 800 U) was detected in the medium incubated with 10 μg of LPS per ml, whereas no activity was detected in LPS-free medium or in the medium incubated with 25 μg of ConA or 10 μg of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). On the other hand, when human epithelial HeLa, T-lymphoma Jurkat, or B-lymphoblastoid Namalwa cells were exposed to 10 μg of LPS per ml or 25 μg of ConA per ml, there was no detectable G-CSF activity in the conditioned media.

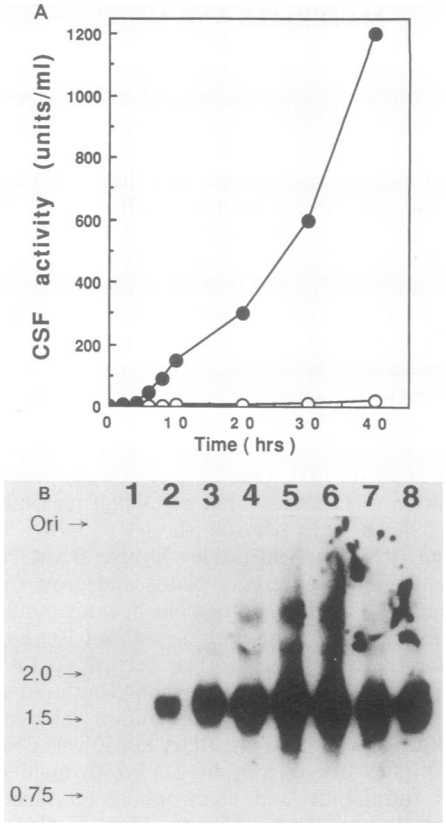

FIG. 2. Induction of the G-CSF gene in mouse BAM3 cells by LPS. (A) Accumulation of CSF activity in the medium of BAM3 cells after treatment with LPS. Cells (1 × 10^6) were seeded onto 10-cm plates and incubated in medium containing 10% FCS with (●) or without (○) 10 μg of LPS per ml. Portions (100 μl) of medium were withdrawn at the times indicated and assayed for CSF activity by using NFS-60 cells as described previously (31). (B) Northern hybridization of RNA from BAM3 cells. Mouse BAM3 cells were incubated with 10 μg of LPS per ml in DMEM containing 10% FCS. At time 0 (lane 1), 6 h (lane 2), 12 h (lane 3), 18 h (lane 4), 24 h (lane 5), 30 h (lane 6), 36 h (lane 7), and 42 h (lane 8), total RNA was prepared from BAM3 cells and analyzed by Northern hybridization with mouse G-CSF cDNA (31) as a probe.

The kinetics of G-CSF production by BAM3 cells were studied by stimulating BAM3 cells with 10 μg of LPS per ml. After a lag period of several hours, G-CSF activity started to accumulate in the medium and reached approximately 1,000 U/ml after 40 h of exposure to LPS (Fig. 2A). More than 90% of the activity could be neutralized by specific rabbit anti-mouse G-CSF antibody, which was prepared by using recombinant mouse G-CSF as an antigen (R. Fukunaga and S. Nagata, manuscript in preparation). RNAs were prepared from BAM3 cells treated with LPS and analyzed by Northern hybridization with mouse G-CSF cDNA (31). No G-CSF mRNA was present in BAM3 cells growing in DMEM containing 10% FCS. On the other hand, when BAM3 cells were treated with LPS, G-CSF mRNA was detected after 6 h and peaked at 24 h (Fig. 2B). Similar results were also obtained by the RNase mapping procedure (M. Nishizawa and S. Nagata, unpublished results).

Previously, we constructed a fusion gene in which about

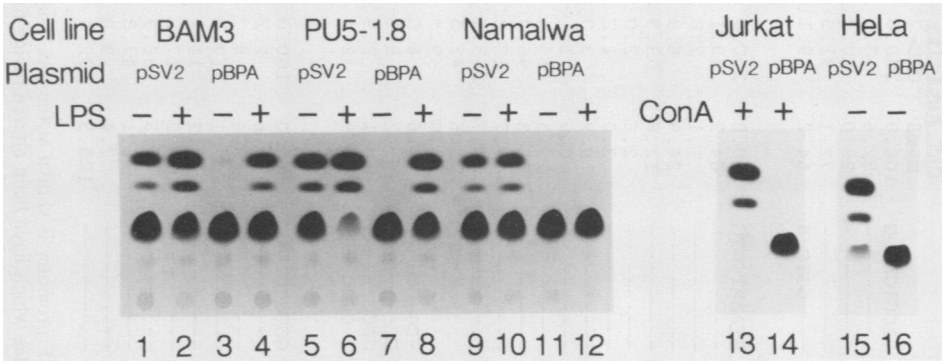

FIG. 3. Expression of the CAT gene in various cell lines. Mouse BAM3 and PU5-1.8 cells and human Namalwa, Jurkat, and HeLa cells were transfected with pBPAcat containing about 300 bp of the G-CSF promoter region fused to the CAT gene. BAM3, PU5-1.8 and Namalwa cells were incubated with or without 10 μg of LPS per ml, whereas Jurkat cells were activated with 25 μg of ConA per ml. As a control for the transfections, pSV2cat was transfected into each cell line. CAT assays were performed as described in Materials and Methods, and the products were separated by thin-layer chromatography. The cells and the plasmids used for transfection are shown above the lanes. Plus and minus denote the presence or absence of LPS or ConA.

300 nucleotides of mouse G-CSF promoter region was placed upstream of the CAT gene (Nishizawa et al., in press). This plasmid DNA was introduced into BAM3, PU5-1.8, HeLa, Jurkat, and Namalwa cells and exposed to 10 μg of LPS or 25 μg of ConA per ml. When the pSV2-CAT gene was introduced into these cells, the CAT activity was detected independently of LPS or ConA stimulation. On the other hand, the mouse G-CSF promoter could induce CAT expression when BAM3 and PU5-1.8 cells were treated with LPS (Fig. 3). This activation was specific to LPS, and no induction of the CAT gene was observed on treatment of cells with ConA or TPA. Furthermore, mouse G-CSF promoter was inert in HeLa, Namalwa, and Jurkat cells, even when the cells were treated with LPS or ConA.

Multiple regulatory sequences in mouse G-CSF promoter. By using a set of linker-scanning mutants and internal-deletion mutants, it was previously shown that at least three regulatory elements in the mouse G-CSF promoter are responsible for the constitutive expression of G-CSF in human carcinoma cells (Nishizawa et al., in press). These elements are GPE1, GPE2, and GPE3, which extend from −196 to −165, from −116 to −105, and from −95 to −64, respectively. To examine whether similar elements are involved in the LPS-inducible expression of the G-CSF gene, the same sets of linker-scanning mutants and internal-deletion mutants were introduced into BAM3 cells, and their promoter activities were assayed by CAT expression. The relative CAT activity of each linker-scanning mutant in BAM3 cells is depicted in Fig. 4, together with the previous results obtained with human squamous carcinoma CHU-2 cells. No plasmid containing linker-scanning mutations promoted CAT expression unless the cells were treated with LPS. Linker-scanning mutations in GPE1 (LS-5 to LS-12) reduced the promoter activity by 4- to 100-fold. In GPE1, not only the consensus CSF box of GAGATTCCCC (LS-5 and LS-6) but also the sequence downstream of the CSF box (LS-7 to LS-12) was essential for the inducible expression of the G-CSF gene. Similarly, mutations in GPE2 (LS-17 to LS-20) diminished the inducible transcription of the G-CSF promoter up to 25- to 100-fold. In GPE3, one linker-scanning mutant (LS-27) had no effect on the constitutive expression in CHU-2 cells, whereas the inducible expression in BAM3 cells was reduced to 6% of the level in the wild-type promoter. Otherwise, mutations in GPE3 had a similar effect on promoter activities in both CHU-2 cells and BAM3 cells. These results indicate that essentially the same regulatory elements in the G-CSF promoter are required for the LPS-inducible expression of the G-CSF gene in macrophages as well as for its ectopic constitutive expression in human carcinoma cells.

To confirm the above results, the mutants with internal deletion mutations of each regulatory element were transfected into BAM3 cells. The deletions from −209 to −277 (INT-1) or from −120 to −155 (INT-5) had no significant effect on the G-CSF promoter activity (Fig. 5). On the other hand, deletions of GPE1 (INT-2, INT-3, and INT-4), GPE2 (INT-6) and GPE3 (INT-7) reduced CAT activity to 0.5 to 10% of that of the wild-type promoter. These results indicate that the three regulatory elements in the G-CSF promoter which were previously identified in CHU-2 cells are also involved in the inducible expression of the G-CSF gene in mouse macrophage BAM3 cells.

Reiterated GPE1 confers the LPS-inducible expression of the CAT gene. To study the contribution of each regulatory element to the LPS induction of the G-CSF gene in macrophages, synthetic oligonucleotides containing the regulatory element were prepared as described in Materials and Methods. The oligonucleotide segment from −119 to −148, on which mutations do not reduce the promoter activity, was also synthesized as a control and is referred to as GPE4. The oligonucleotide sequence was reiterated four times and placed upstream of the −67 mouse G-CSF promoter containing the TATA box. The elements were inserted in the correct orientation or in the reverse orientation, and two tandem polyadenylation sites from simian virus 40 were located upstream of the elements to reduce the readthrough transcription from pBR322 vector.

The structures of the reporter genes thus constructed are illustrated in Fig. 6. These hybrid genes were introduced into a variety of cells, including BAM3, PU5-1.8, Namalwa, Jurkat, and HeLa cells. The G-CSF promoter, up to −67 (pBPA-TATA), did not promote CAT expression in all types of cells tested (Table 1). However, the expression of the reporter gene linked to the four copies of GPE1 (pTA-E14 and pTA-E14R) was highly inducible by LPS in BAM3 and PU5-1.8 cells. The four copies of GPE1 operate in either orientation, although the efficiency of the induction seems to be enhanced when the element is placed in the opposite orientation relative to the promoter sequence. The effect of GPE1 was specific in macrophage cell line BAM3 and

	BamHI				-250					CAT Activity BAM3	CAT Activity CHU-2
Wild type	GGATCCAGA	TCCAACACCC	TGCAGCGATT	CAGGCCTGGG	TGTGGCTGGA	AGAGAGGAAG	AGAGTTTGAG	GGGGGGACAA	GACGTCAAGG	100.0	100.0
LS-1	---------	---GGTCGA-	GA-C------	---------	---------	---------	---------	---------	---------	82.6	50.6
LS-2	---------	---------	---------	---------	G--C-AC-AC	C---------	---------	---------	---------	25.0	20.0
LS-3	---------	---------	---------	---------	---------	---------	--------G-	TC-ATCGACC	---------	72.1	81.5
LS-4	---------	---------	---------	---------	---------	---------	---------	---------	----GGTC-A	64.2	49.1

	-200				-150						
Wild type	AAGGAACAGA	GATTCCCCGA	TTTCACAAAA	ACTTTCGCAA	ACAGCTTTTC	CCCAACCCCC	TGCATTGTCC	TGAGCTATCA	TCAAATTTGC	ATAAATCCTG	100.0	100.0
LS-4	CC--------	---------	---------	---------	---------	---------	---------	---------	---------	---------	64.2	49.1
LS-5	----TCG-CG	ACC-------	---------	---------	---------	---------	---------	---------	---------	---------	1.1	2.8
LS-6	---------G	-TCGA-----	---------	---------	---------	---------	---------	---------	---------	---------	24.9	6.9
LS-7	---------	---------G	-CGAC-----	---------	---------	---------	---------	---------	---------	---------	12.8	3.3
LS-8	---------	---------G	G-CG--G-CC	---------	---------	---------	---------	---------	---------	---------	2.6	2.0
LS-9	---------	---------	-GGTCG-CC-	---------	---------	---------	---------	---------	---------	---------	3.0	1.2
LS-10	---------	---------	--GGT-G-CC	---------	---------	---------	---------	---------	---------	---------	4.9	3.0
LS-11	---------	---------	----GGTCG-	CGACC-----	---------	---------	---------	---------	---------	---------	0.9	1.1
LS-12	---------	---------	---------	GG-CGACG-C	C---------	---------	---------	---------	---------	---------	19.3	19.5
LS-13	---------	---------	---------	---------	GGTCGACC--	---------	---------	---------	---------	---------	75.3	96.9
LS-14	---------	---------	---------	---------	-----GG-CG	-GACC-----	---------	---------	---------	---------	37.7	22.2
LS-15	---------	---------	---------	---------	---------	---------	---GG-CG-G	ACC-------	---------	---------	70.7	273.4
LS-16	---------	---------	---------	---------	---------	---------	---------	G-TCGACGAC	C---------	---------	73.6	42.5
LS-17	---------	---------	---------	---------	---------	---------	---------	---------	-----GGCC-	---------	1.0	9.5
LS-18	---------	---------	---------	---------	---------	---------	---------	---------	-----GG-CG	-CG-CC----	0.6	6.0
LS-19	---------	---------	---------	---------	---------	---------	---------	---------	---------G	CC--------	1.2	3.5
LS-20	---------	---------	---------	---------	---------	---------	---------	---------	---------	GGCC------	4.1	2.9
LS-21	---------	---------	---------	---------	---------	---------	---------	---------	---------	--GGCC----	70.9	32.4

	-100				SmaI -50				-1			
Wild type	GGAAGTTATT	ACTAAGCCTG	AGTTGCCCCA	GCCCCAGGTA	ATTTCCTCCC	GGGGCCTTGA	TGGCTTTATG	TATAAAGGCC	CCCTGGAGCT	GGGCCCTGGC	100.0	100.0
LS-22	-----GGTCG	--G-CC----	---------	---------	---------	---------	---------	---------	---------	---------	0.6	2.2
LS-23	--------GG	T-G-CC----	---------	---------	---------	---------	---------	---------	---------	---------	3.4	4.7
LS-24	---------	---GGT-GC-	-CC-------	---------	---------	---------	---------	---------	---------	---------	12.1	17.9
LS-25	---------	---------	---G-T-G-G	A---------	---------	---------	---------	---------	---------	---------	13.2	11.2
LS-26	---------	---------	---------G	-T-GATC-AC	C---------	---------	---------	---------	---------	---------	13.0	17.2
LS-27	---------	---------	---------	--------GG	TCGA------	---------	---------	---------	---------	---------	5.3	136.9
LS-28	---------	---------	---------	---------	-GG--GCGA-	C---------	---------	---------	---------	---------	16.1	47.8
LS-29	---------	---------	---------	---------	--GGTCGA--	---------	---------	---------	---------	---------	7.4	9.0
LS-30	---------	---------	---------	---------	---------	C--T-GACCG	---------	---------	---------	---------	125.3	102.4

FIG. 4. Effect of linker-scanning mutations on the inducible expression of the G-CSF promoter. The nucleotide sequence of the mouse G-CSF promoter is shown at the top. The NF-κB-like CSF box, the octamer transcription factor-binding site, and the TATA box are underlined. In each linker-scanning mutant, nucleotides which have been altered owing to sequence substitutions of the linker are indicated. Plasmids carrying each mutated promoter were transfected into BAM3 cells, and the cells were treated with 10 μg of LPS per ml. CAT activities obtained with each mutant are shown as a percentage of that of the wild-type promoter. For comparison, CAT activities obtained from each mutant in human squamous carcinoma CHU-2 cells (Nishizawa et al., in press) are also shown.

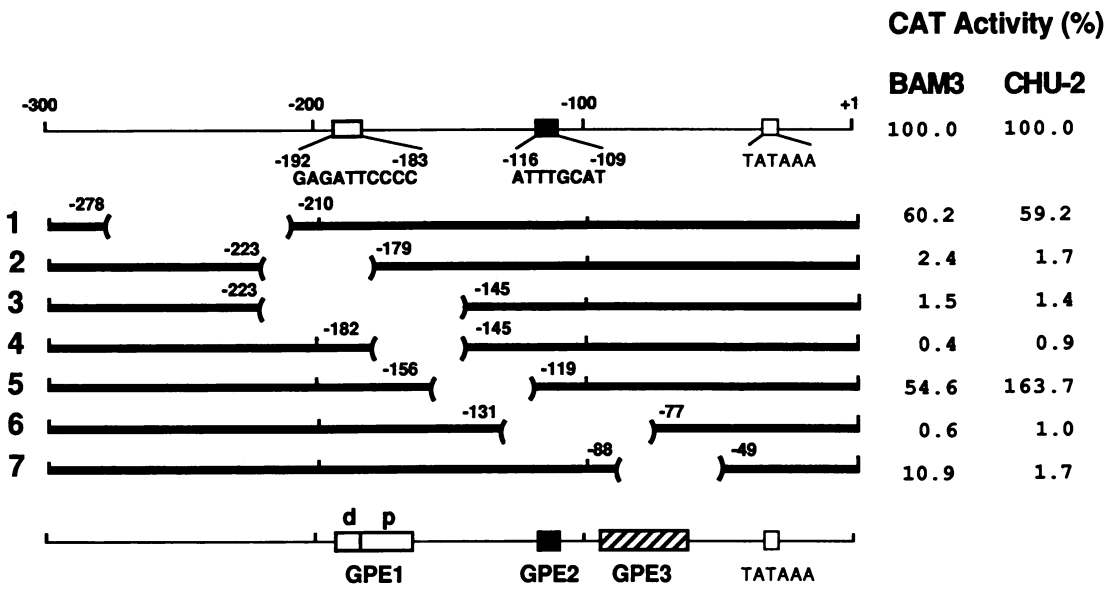

FIG. 5. Promoter activities of internal deletion mutants of the G-CSF promoter in LPS-stimulated BAM3 cells. The top line shows the promoter of mouse G-CSF gene, on which the locations of consensus sequence of the CSF box, the OTF-binding site, and the TATA box are indicated. The deletion endpoints of each internal deletion mutant are indicated above each line. The CAT activities are shown as described in the legend to Fig. 3, together with data obtained from human squamous carcinoma CHU-2 cells (Nishizawa et al., in press). Three regulatory elements (GPE1, GPE2, and GPE3) are depicted in the schematic representation of the promoter shown at the bottom.

macrophage-monocyte cell line PU5-1.8, and no effect was observed in Namalwa, Jurkat, or HeLa cells. On the other hand, the tetramer of the other GPEs, GPE2, GPE3, and GPE4, did not confer inducibility even in BAM3 and PU5-1.8 cells. When the enhancer activities of the dimer, tetramer, and hexamer of GPE1 were examined, it was revealed that GPE1 worked more efficiently in PU5-1.8 cells than in BAM3 cells and that the construct containing the GPE1 hexamer showed the strongest activity in both cell lines (Table 2). Taken together, these data indicate that GPE1 displays the properties of a cell-specific inducible enhancer.

GPE1 comprises two elements, the distal element containing the NF-κB-like CSF box and the proximal element containing the sequence specific for the G-CSF promoter (Fig. 4). To examine which element functions as an inducible element, we synthesized oligonucleotides containing the

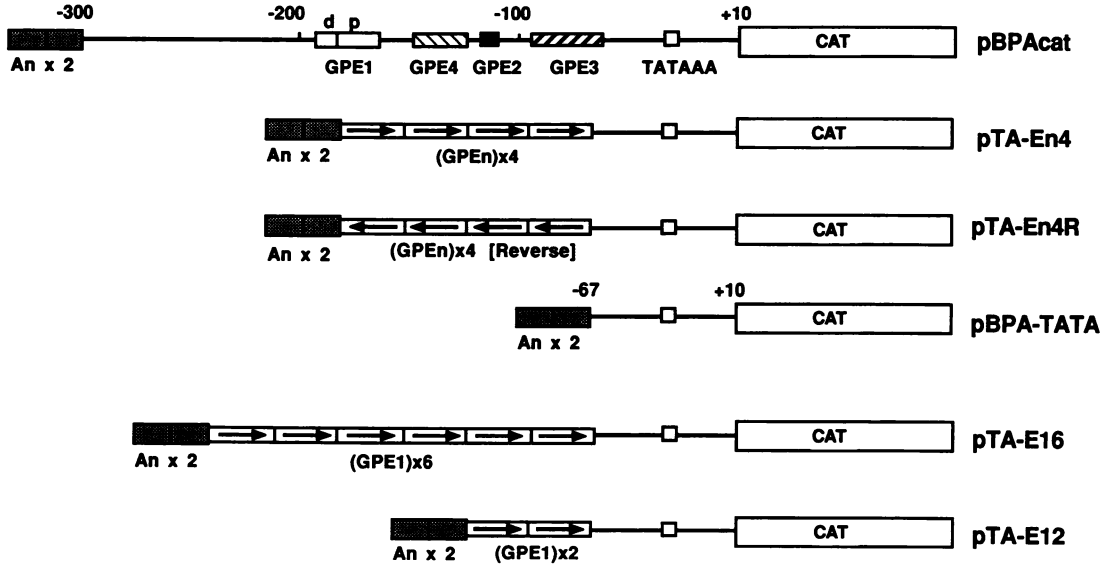

FIG. 6. Construction of expression plasmids containing multiple copies of GPE. The expression plasmid (pBPAcat), in which an approximately 300-bp mouse G-CSF promoter was fused to the CAT gene, is shown at the top. The locations of each regulatory element are indicated on pBPAcat. Mutations in the GPE4 region had no effect on the promoter activity and were used as a reference. The regions indicated by An × 2 represent the polyadenylation site from simian virus 40 arranged in tandem, which prevents readthrough transcription from the upstream region. Four copies of the oligonucleotides containing each regulatory element were inserted upstream of the truncated −67 mouse G-CSF promoter–CAT fusion gene (pBPA-TATA) in the correct orientation (pTA-En4) or the reverse orientation (pTA-En4R). The structures of the reporter genes containing the hexamer or dimer of GPE1 are shown as pTA-E16 or pTA-E12, respectively.

TABLE 1. Enhancer activity of four copies of GPE in various cell lines

Plasmid	Relative CAT activity					
	BAM3 (+LPS)[a]	BAM3 (−LPS)[a]	PU5-1.8 (+LPS)[a]	Namalwa (+LPS)[b]	Jurkat (+ConA)[b]	HeLa[b]
pSV2cat	138.3	87.3	174.0	100.0	100.0	100.0
pBPAcat	100.0	1.1	100.0	0.6	0.4	0.2
pTA-E14	43.2	0.4	110.0	0.6	0.1	0.2
pTA-E14R	89.0	0.4	127.6	0.6	0.1	0.1
pTA-E24	0.8	0.4	1.3	0.6	0.1	0.6
pTA-E24R	1.1	1.0	4.7	0.5	0.3	0.1
pTA-E34	1.1	0.9	0.5	0.6	0.3	0.1
pTA-E34R	2.2	0.7	0.3	ND[c]	0.1	0.1
pTA-E44	0.5	0.3	0.2	0.5	0.1	0.1
pTA-E44R	0.5	0.2	0.1	ND	0.1	0.1
pBPA-TATA	0.4	0.2	0.2	0.4	0.1	0.1
pSV0Acat	0.6	0.3	0.1	0.6	0.1	0.1

[a] The CAT activities are presented as a percentage of that of the wild-type promoter (pBPAcat) in LPS-treated cells.
[b] The CAT activities of pSV2cat were taken as 100%.
[c] ND, Not done.

distal or proximal element and assayed them for enhancer activity as described above. Four copies of the proximal element of GPE1 linked to CAT gene significantly stimulated CAT expression, whereas no stimulating activity was observed with four copies of the distal element (Table 3).

Cellular factors that bind to GPE1. To identify cellular factors that could mediate inducibility through GPE1, we carried out a gel retardation assay with a GPE1 probe spanning the region from −193 to −152. Several complexes were detected in the nuclear extract prepared from BAM3 cells (Fig. 7A, lane 2). The specificity of the binding reaction was then examined by competitive binding with a variety of DNA fragments. All of complexes were competed for specifically by the DNA fragments containing either GPE1 or its proximal part (Fig. 7A, lanes 3 and 5). On the other hand, no competition was observed with DNA fragments spanning the distal part of GPE1 (lane 4) or GPE4 (lane 8), although DNA fragments containing GPE2 or GPE3 competed weakly for complex formation. These results indicate that some nuclear proteins specifically bind to the proximal element of GPE1. To examine the possibility that some nuclear proteins are induced or suppressed after treatment with LPS, we performed a gel retardation assay by using nuclear extracts from LPS-induced BAM3 and PU5-1.8 cells. Almost the same set of shifted bands was observed when using nuclear extracts either from noninduced cells or from LPS-induced cells, although the intensity of one band was stronger with nuclear extract from LPS-treated BAM3 cells than that with nuclear extract from untreated cells (Fig. 7B, lanes 2 and 3). Furthermore, when nuclear extracts from human CHU-2 and HeLa cells were examined by the gel retardation assay, similar shifted bands were detected (lanes 6 and 7). In this case, one band visible with nuclear extract from CHU-2 cells was very weak with nuclear extract from HeLa cells, whereas another complex formed with the extract from HeLa cells was missing with the extract from CHU-2 cells.

DNase I and neocarzinostatin footprint analyses were then carried out to map the region where the nuclear factors bind. Nuclear proteins prepared from PU5-1.8 or BAM3 cells either treated with LPS or not treated were incubated with a ^{32}P-labeled DNA fragment containing GPE1 and digested with DNase I or neocarzinostatin, and the cleavage products were displayed on a denaturating polyacrylamide gel. The region from −186 to −161 on the coding strand was protected by nuclear factors from DNase I and neocarzinostatin digestion (Fig. 7C). Nuclear extracts prepared from either LPS-treated cells or untreated cells exhibited a similar effect on the protection. Furthermore, when [^{32}P]DNA fragment labeled on the noncoding strand was used as a probe, the same region was protected by nuclear factors (data not shown). These results indicate that nuclear factors in BAM3 cells and PU5-1.8 cells bind to the proximal part of GPE1, but not to the NF-κB-like consensus CSF box.

DISCUSSION

In the present work, we have shown that several mouse macrophage or macrophage-monocyte cell lines can be stimulated by LPS to produce G-CSF (Fig. 2). These cell lines

TABLE 2. Effect of the GPE1 dimer, tetramer, and hexamer on promoter activity

Plasmid	Relative CAT activities[a,b]	
	BAM3	PU5-1.8
pBPAcat	100.0	100.0
pTA-E16	87.3	107.3
pTA-E14	45.3	86.8
pTA-E12	1.6	21.5
pBPA-TATA	0.4	0.2

[a] Cells were treated with 10 μg of LPS per ml for 48 h.
[b] The CAT activities are expressed as a percentage of that of the wild-type promoter (pBPAcat).

TABLE 3. Enhancer activity of the proximal part of GPE1

Plasmid	Relative CAT activities[a,b]	
	BAM3	PU5-1.8
pBPAcat	100.0	100.0
pTA-E14	39.6	101.2
pTA-E14R	133.9	111.5
pTA-E1d4	0.2	0.2
pTA-E1d4R	0.3	0.1
pTA-E1p4	12.2	14.5
pTA-E1p4R	30.4	22.5
pBPA-TATA	0.5	0.2
pSV0Acat	0.2	0.3

[a] Cells were treated with 10 μg of LPS per ml for 48 h.
[b] The CAT activities are expressed as a percentage of that of the wild-type promoter (pBPAcat).

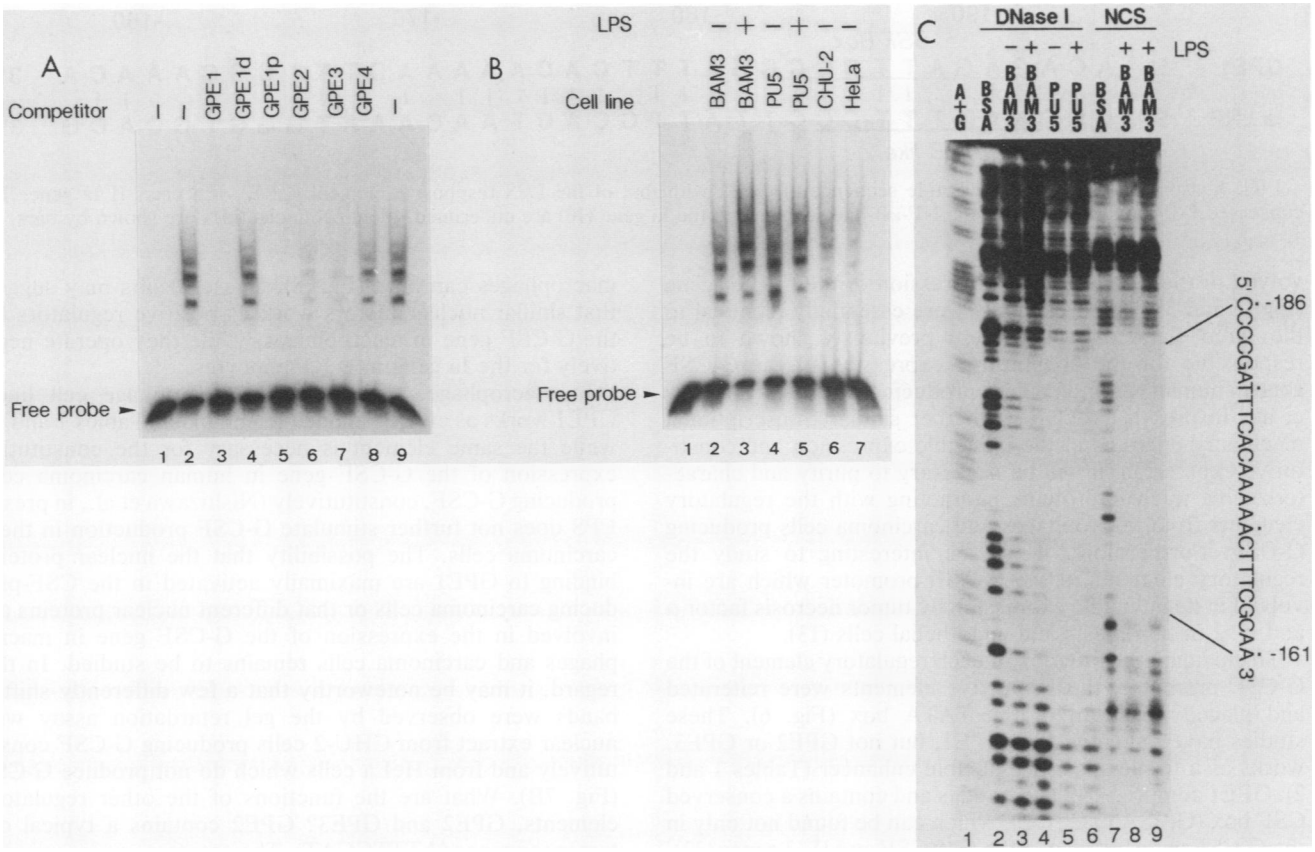

FIG. 7. Binding of nuclear factors to GPE1. (A) Gel retardation assays for the detection of factors that specifically bind to GPE1. Nuclear extracts (2 μg) from uninduced BAM3 cells were incubated with 2 fmol of the ^{32}P-labeled *Hin*dIII-*Xho*I fragment of pGPE1-1 (lanes 2 to 9). As indicated above each lane, about a 160-fold excess of unlabeled DNA fragments prepared from pGPEn-4 containing four copies of each GPE was included in the reaction mixture (lanes 3 to 8) as competitor DNAs. (B) Gel retardation assays with different nuclear extracts. Nuclear extracts were prepared from BAM3 (lanes 2 and 3), PU5-1.8 (lanes 4 and 5), CHU-2 (lane 6), and HeLa (lane 7) cells. BAM3 and PU5-1.8 cells were harvested without treatment (lanes 2 and 4) or after treatment with 10 μg of LPS per ml for 12 h (lanes 3 and 5). A 2-μg (BAM3 and PU5-1.8) or 5-μg (CHU-2 and HeLa) portion of nuclear extracts from each cell line was incubated with 2 fmol of ^{32}P-labeled *Hin*dIII-*Xho*I fragment of pGPE1-1 and analyzed by electrophoresis on a 4% polyacrylamide gel. (C) Footprint analysis of nuclear factor-binding interactions with GPE1. ^{32}P-labeled DNA probes were incubated with 20 μg of nuclear extract prepared from untreated or LPS-treated BAM3 or PU5-1.8 cells and subjected to partial DNase I (lanes 2 to 6) or neocarzinostatin (lanes 7 to 9) digestion. For the neocarzinostatin digestion, incubation was carried out for 10 min (lanes 7 and 8) or 30 min (lane 9). As a control, 20 μg of bovine serum albumin was used instead of nuclear extract and subjected to partial digestion with DNase I (lane 2) or neocarzinostatin (lane 7). Lane 1 is a sequence marker prepared by A+G modification by the method of Maxam and Gilbert (16).

respond specifically to LPS, and no production of G-CSF was observed by other agents such as ConA or TPA. These results are in contrast to the observations of Vellenga et al. (34), who showed that TPA can stimulate human blood monocytes to express the G-CSF gene. It is possible that these different results were due to the different systems between the established cell lines and primary monocytes. However, since activated T lymphocytes can stimulate the release of G-CSF from blood monocytes (24), the effect of TPA on the expression of the G-CSF gene in blood monocytes is probably an indirect effect via T lymphocytes contained in the monocyte preparation.

Recently, it was suggested that G-CSF gene expression in human monocytes is regulated primarily at the posttranscriptional level (7). In contrast, a hybrid gene consisting of a 300-bp mouse G-CSF promoter and a CAT gene was inert in macrophage and macrophage-monocyte cells in the transient assay system (Fig. 3). The promoter was strongly activated by treatment of the cells with LPS. When the approximately 3.5-kilobase 5′-flanking region of the mouse G-CSF promoter was linked to the CAT gene, similar stimulation of CAT gene expression was observed with LPS (M. Nishizawa and S. Nagata, unpublished results). These results indicate that the induction of the G-CSF gene by LPS in macrophage and macrophage-monocyte cell lines is regulated at the transcriptional level. A number of transiently expressed genes have an ATTTA motif in their 3′-noncoding regions of mRNA, and the motif has been shown to contribute to the instability of the mRNA (30). Since the G-CSF mRNA contains seven copies of the ATTTA sequence in the 3′-noncoding region, it is likely that the expression of G-CSF genes in macrophage and macrophage-monocyte cell lines is further modulated at the posttranscriptional level by stabilizing the mRNA. In this regard, it is noteworthy that the expression of the G-CSF gene by tumor necrosis factor α in mesenchymal cells is regulated at both transcriptional and posttranscriptional levels (13).

By analyzing a set of linker-scanning mutants and internal-deletion mutants of the G-CSF promoter, we showed that at least three regulatory elements in the promoter were in-

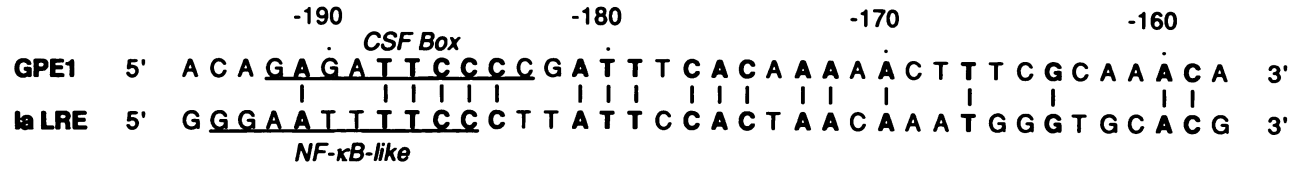

FIG. 8. Comparison of the nucleotide sequence of GPE1 with that of the LPS responsive element (LRE) of a class II Ia gene. The conserved CSF box in GPE1 and the NF-κB-like sequence in the Ia gene (10) are underlined. Identical nucleotides are shown by bars.

volved in the LPS-inducible expression of the G-CSF gene (Fig. 4 and 5). These elements were essentially identical to the three elements which were previously shown to be responsible for the constitutive expression of the G-CSF gene in human carcinoma cells producing G-CSF (Nishizawa et al., in press). To clarify whether similar transcriptional machinery operates in the inducible expression and constitutive expression, it will be necessary to purify and characterize the nuclear proteins interacting with the regulatory elements from macrophages and carcinoma cells producing G-CSF. Furthermore, it will be interesting to study the regulatory elements in the G-CSF promoter which are involved in its inducible expression by tumor necrosis factor α and IL-1 in fibroblasts and endothelial cells (13).

To elucidate the function of each regulatory element of the G-CSF promoter, the respective elements were reiterated and placed upstream of the TATA box (Fig. 6). These studies have indicated that GPE1, but not GPE2 or GPE3, works as a tissue-specific inducible enhancer (Tables 1 and 2). GPE1 comprises 42 nucleotides and contains a conserved CSF box (GAGATTCCCC), which can be found not only in the G-CSF gene but also in the GM-CSF and IL-3 genes (32). Since the CSF box is related to the NF-κB element (GGGG GATTTCC), which functions as an LPS-inducible enhancer for the immunoglobulin κ gene in pre-B cells (1), it was expected that the CSF box alone works as an LPS-inducible enhancer in macrophages. However, surprisingly, the reiterated CSF box (the distal element of GPE1) did not mediate the LPS-induced CAT gene expression, while the proximal element of GPE1 (31 nucleotides long) could significantly stimulated CAT expression after the treatment of cells with LPS (Table 3). Furthermore, nuclear proteins prepared from BAM3 or PU5-1.8 cells did bind specifically to the proximal part of GPE1 (Fig. 7). These results suggest that the proximal element of GPE1 plays an important role in the inducible expression of the G-CSF gene in macrophages. However, several shifted bands detected by the gel retardation assay showed no clear differences whether the nuclear extracts were prepared from untreated or LPS-treated macrophage cell lines (Fig. 7B). These results may suggest that there are several nuclear proteins which bind to the proximal part of GPE1 and that only minor changes of nuclear proteins are responsible for the inducible expression of the G-CSF gene in macrophages. To clarify these points, it will be necessary to isolate the cDNAs which code for the nuclear proteins binding to GPE1.

Recently, Gravallese et al. (10) reported that an LPS-inducible protein can bind to a region of the promoter in the class II histocompatibility Ia gene, which is down regulated by LPS in B-cells. Although the region contains the NF-κB-like element, the actual protein-binding site was distinct from the NF-κB element, and a new LPS-responsive sequence was proposed (10). When the sequence of GPE1 was aligned with the sequence in the Ia gene, a significant degree of homology was found not only in the CSF box but also in the downstream sequences on which nuclear proteins from macrophages can bind (Fig. 8). These results may suggest that similar nuclear factors work as positive regulators for the G-CSF gene in macrophages, while they operate negatively for the Ia gene in B lymphocytes.

In macrophages and monocyte-macrophage cell lines, GPE1 works as an LPS-inducible enhancer (Tables 1 and 2), while the same element is necessary for the constitutive expression of the G-CSF gene in human carcinoma cells producing G-CSF, constitutively (Nishizawa et al., in press). LPS does not further stimulate G-CSF production in these carcinoma cells. The possibility that the nuclear proteins binding to GPE1 are maximally activated in the CSF-producing carcinoma cells or that different nuclear proteins are involved in the expression of the G-CSF gene in macrophages and carcinoma cells remains to be studied. In this regard, it may be noteworthy that a few differently shifted bands were observed by the gel retardation assay with nuclear extract from CHU-2 cells producing G-CSF constitutively and from HeLa cells which do not produce G-CSF (Fig. 7B). What are the functions of the other regulatory elements, GPE2 and GPE3? GPE2 contains a typical octamer sequence (ATTTGCAT). The octamer sequence in the promoter of immunoglobulin genes works as a B-cell-specific promoter element, and the octamer-binding protein (OTF-2) which is specifically expressed in B lymphocytes was identified (27) and its cDNA was molecularly cloned (20, 26). Since the G-CSF gene is expressed not only in macrophages and monocytes but also in CSF-producing tumor cells from several origins, OTF-1, which is believed to be expressed ubiquitously, is likely to function on the G-CSF gene. Purification and characterization of the octamer-binding proteins from macrophages and CSF-producing carcinoma cells may clarify these questions. GPE3 consists of about 40 nucleotides, and a similar sequence has not been found in the promoters of other mammalian genes (36). To study its function, it will be necessary to examine the element in more detail and to analyze the proteins binding to GPE3.

ACKNOWLEDGMENTS

We thank K. Ohki for providing mouse BAM3 cells. We are grateful to R. Watanabe-Fukunaga for construction and preparation of plasmid DNAs.

This work was performed in part through Special Coordination Funds of the Science and Technology Agency of the Japanese Government, and supported in part by grants-in-aid from the Ministry of Education, Science and Culture of Japan.

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