MOLECULAR AND CELLULAR BIOLOGY, May 1988, p. 1979–1984
0270-7306/88/051979–06$02.00/0

Vol. 8, No. 5

Characterization of the Human Granulocyte-Macrophage Colony-Stimulating Factor Promoter Region by Genetic Analysis: Correlation with DNase I Footprinting

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Received 9 October 1987/Accepted 5 February 1988

T-cell activation induces expression of the hematopoietic growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF). To define the molecular events involved in the induction of GM-CSF gene expression more clearly, we prepared and analyzed deletion mutants of GM-CSF promoter recombinant constructs. The results localized inducible expression to a 90-base-pair region (−53 to +37) which is active in uninfected and human T-cell leukemia virus-infected T-cell lines but not in resting or mitogen-stimulated B cells. DNase I footprinting experiments revealed protection of sequences contained within this region, including a repeated nucleotide sequence, CATT(A/T), which could serve as a core recognition sequence for a cellular transcription factor. Upstream of these GM-CSF promoter sequences is a 15-base-pair region (−193 to −179) which has negative regulatory activity in human T-cell leukemia virus-infected T cells. These studies revealed a complex pattern of regulation of GM-CSF expression in T cells; positive and negative regulatory sequences may play critical roles in controlling the expression of this potent granulopoietin in the bone marrow microenvironment and in localized inflammatory responses.

Colony-stimulating factors (CSF) play an important role in controlling the proliferation of hematopoietic progenitor cells and enhancing the functional activities of mature myeloid effector cells (reviewed in reference 18). Human granulocyte-macrophage CSF (GM-CSF) stimulates the formation of neutrophil, neutrophil-macrophage, macrophage, and eosinophil colonies from normal human bone marrow and, under certain conditions, can increase the formation of erythroid and megakaryocyte colonies (19, 31, 35). GM-CSF also enhances many of the host defense activities of mature neutrophils, monocytes, and eosinophils (5, 6, 9, 12, 32, 37–39). In vivo activity of recombinant GM-CSF has been demonstrated in several clinical trials in humans (11, 36).

Expression of GM-CSF is tightly regulated. Activated but not resting T lymphocytes produce GM-CSF (40), and certain fibroblasts and endothelial cells can be stimulated by tumor necrosis factor or interleukin 1 (IL-1) to produce GM-CSF (22, 43). Recently, GM-CSF production by stimulated mouse peritoneal macrophages has been observed (34).

We have been interested in the molecular mechanisms involved in the control of GM-CSF expression after T-cell activation. The MLA144 gibbon ape and Jurkat human T-cell lines can be stimulated to produce GM-CSF and can be used to study induction of GM-CSF expression (40; unpublished data). Human T-cell leukemia virus (HTLV)-infected T-cell lines produce GM-CSF constitutively but can also be stimulated with phytohemagglutinin (PHA) and phorbol diesters (such as phorbol myristate acetate [PMA]) to increase GM-CSF production 5- to 10-fold (2, 41), providing an ideal opportunity to study both constitutive and induced expression of GM-CSF.

We previously identified a 660-base-pair (bp) region of 5′-flanking GM-CSF DNA which contained regulatory sequences involved in increasing the expression of GM-CSF after T-cell activation in an HTLV-infected T-cell line (S-LB-I) (2). We now show that this region is also active in the HTLV-uninfected MLA144 and Jurkat T-cell lines, and by using deletion mutant analysis we identified both positive and negative regulatory elements active in the control of GM-CSF expression. We also performed DNase I footprinting experiments to look for DNA-protein interactions in the GM-CSF promoter. These experiments demonstrate DNase I protection, consistent with the binding of nuclear protein(s), in the GM-CSF 5′-flanking sequences shown by genetic analysis to contain positive regulatory activity.

MATERIALS AND METHODS

Transfection procedure. Cells were placed in fresh medium on the day before transfection and washed twice with serum-free Scove modified Dulbecco medium (IMDM) before the procedure. Cells (2×10^7) were incubated in 2 ml of serum-free IMDM with DEAE-dextran (0.25 mg/ml)–0.05 M Tris hydrochloride (pH 7.4)–0.1 mM chloroquine–10 μg of DNA per ml for 2 h at 37°C. The cells were then washed with serum-free IMDM and transferred to Falcon tissue culture plates containing 15 ml of IMDM with 10% (or 20%) fetal bovine serum. At 24 h later, the cells were stimulated with 0.5% PHA and 10 ng of PMA per ml. At 20 h after stimulation, the cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity as previously described (8).

Construction of plasmids. The +pCSFp1 construction, containing 660 bp of 5′-flanking GM-CSF sequences linked to the *cat* gene, was linearized at the *Hin*dIII site (−626, relative to the GM-CSF cap site) (2). Limited BAL 31 exonuclease digestion was performed for various lengths of time, and the extent of the deletion was checked by agarose gel electrophoresis. The linear fragments were ligated over-

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night and used to transform *Escherichia coli* HB101 bacteria. Plasmids were prepared by standard procedures and sequenced to determine the 5′ end of the remaining GM-CSF DNA sequences (17). Plasmid −53 was obtained by digesting +pCSFp1 with *Hin*dIII and *Bst*EII, treating it with the Klenow fragment of DNA polymerase I to create blunt ends, and performing blunt-end ligation. This resulted in deletion of base pairs −626 to −54 without deletion of plasmid sequences.

Preparation of nuclear extracts. Crude nuclear extracts were prepared from 5 to 10 liters of S-LB-I and MLA144 cells at a cell density of 0.8×10^6 to 1.2×10^6 cells per ml by the method of Shapiro et al. (28). The cells were fed on the day before harvesting and then either stimulated with 1% PHA–50 ng of PMA per ml for 4 to 6 h or treated with diluent control. The extracts were dialyzed twice against >200 volumes of nuclear dialysis buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 20% [vol/vol] glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EDTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 2 mM dithiothreitol) for 90 min each time. Nuclear extract protein concentrations, generally 15 to 25 mg of protein per ml, were determined by the Bio-Rad protein assay. The nuclear extracts were frozen in aliquots at −70°.

DNase I footprinting experiments. The DNase I digestion procedures were performed by the method of Dynan and Tjian (4). DNA-binding reactions were performed on ice for 15 min in 50 μl of Tris buffer containing approximately 10 fmol of end-labeled DNA fragment, 1 μg of unlabeled carrier DNA [poly(dI-dC) in 10 mM Tris hydrochloride (pH 7.9), 1 mM EDTA], and 0, 25, 100, or 200 μg of nuclear extract. Then 50 μl of a 5 mM $CaCl_2$–10 mM $MgCl_2$ solution and 50 to 200 ng of DNase I (Worthington Diagnostics) per ml (final concentration) were added. After 1 min at room temperature, the reactions were terminated by addition of 90 μl of STOP mixture (200 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate, 1.7 μg of yeast tRNA per ml). The samples were extracted with phenol and chloroform, ethanol precipitated, and loaded onto an 8% denaturing polyacrylamide gel.

The labeled fragment used for DNase I footprinting was prepared by inserting a 292-bp *Bbv*I fragment of GM-CSF sequences into the *Bam*HI site of pUC18. To footprint the coding strand, this plasmid (pF) was end labeled with γ-^{32}P at the *Bam*HI site, and a *Bam*HI-*Mst*II fragment (base pairs −218 to +46) was gel isolated. To footprint the noncoding strand of the GM-CSF promoter, the pF plasmid was end labeled with γ-^{32}P at the *Avr*II site, and an *Avr*II-*Bam*HI fragment (base pairs −145 to +46) was gel isolated.

RESULTS

Identification of GM-CSF promoter activity in uninfected T-cell lines. Our previous studies showed that a 660-bp fragment of 5′-flanking GM-CSF sequences (+pCSFp1; Fig. 1) could direct increased expression of CAT activity after PHA and PMA stimulation of the HTLV-I-infected S-LB-I cell line (2). This plasmid is not active in resting B cells or in B cells treated with pokeweed mitogen, lipopolysaccharide, or PMA (2; unpublished data).

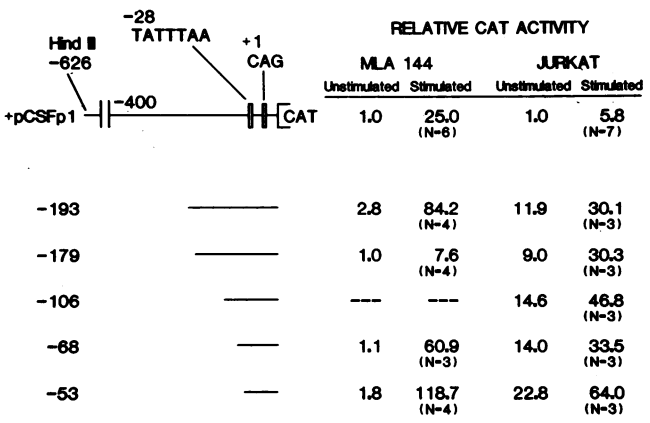
To determine whether these 5′ sequences were active in HTLV-uninfected T cells, we transfected +pCSFp1 into Jurkat and MLA144 cells. Neither cell line produced detectable GM-CSF mRNA or protein in the absence of stimulation (data not shown), and consistent with this observation, no CAT activity above background was seen in either unstimulated MLA144 or Jurkat cells (0.3% conversion for both MLA144 and Jurkat cells, with a background activity of 0.5% directed by a promoterless construct; data not shown).

Activation of both cell lines resulted in detectable expression of the endogenous GM-CSF gene and detectable CAT activity. A 10- to 40-fold increase in CAT activity was observed after stimulation of MLA144 cells, similar to the increase in GM-CSF protein levels in MLA144 cell-conditioned medium (from <2 to 10 to 37ng/ml, as measured by radioimmunoassay). Thus, similar activation of both the transfected and endogenous genes occurred. In Jurkat cells, a 3- to 10-fold increase in CAT activity (over background counts) was seen, though GM-CSF levels in the conditioned medium remained undetectable by our radioimmunoassay (<2 ng/ml).

To identify functional regions within this 660-bp region, a series of recombinant constructs containing deletions at the 5′ end was prepared (Fig. 1). The most extensive deletion mutant, plasmid −53, which contains 53 bp upstream of the GM-CSF cap site and 37 bp downstream, contained sufficient 5′ regulatory sequences to retain full inducible promoter activity in MLA144 and Jurkat cells. Although the fold induction in CAT activity seen with plasmid −53 was similar to that observed for +pCSFp1 in Jurkat cells, the 5′ deletion mutants consistently directed a greater amount of CAT activity than did the +pCSFp1 plasmid in unstimulated Jurkat cells but not in unstimulated MLA144 cells (Fig. 1). This implies the presence of a negative regulatory element, located between base pairs −626 and −193, which, when present, prevents basal expression of CAT activity in Jurkat cells.

Two control plasmids were used for these experiments: −pCSFp1, which contains 660 bp of 5′ GM-CSF sequences linked to the *cat* gene in the reverse orientation, and BUG-CAT (kindly provided by Julian Banerji), which contains the simian virus 40 early promoter and a transcriptionally inactive DNA fragment linked to the *cat* gene. No increase in CAT activity was seen after T-cell stimulation for either of these plasmids in the cell lines tested (data not shown).

Identification of a negative regulatory element in the GM-



	RELATIVE CAT ACTIVITY			
	MLA 144		JURKAT	
	Unstimulated	Stimulated	Unstimulated	Stimulated
+pCSFp1	1.0	25.0 (N=6)	1.0	5.8 (N=7)
-193	2.8	84.2 (N=4)	11.9	30.1 (N=3)
-179	1.0	7.6 (N=4)	9.0	30.3 (N=3)
-106	---	---	14.6	46.8 (N=3)
-68	1.1	60.9 (N=3)	14.0	33.5 (N=3)
-53	1.8	118.7 (N=4)	22.8	64.0 (N=3)

FIG. 1. Structure and regulatory activity of GM-CSF-CAT deletion mutants. Plasmid +pCSFp1 was digested with either BAL 31 exonuclease or restriction endonucleases, resulting in plasmids −193, −179, −106, −68, and −53. The CAT activity generated by these constructs was measured in both unstimulated and stimulated MLA144 and Jurkat cells and is given as a ratio relative to the CAT activity generated by the +pCSFp1 plasmid in unstimulated cells. The numbers represent an average of at least three experiments (the exact numbers of experiments are shown in parentheses).

CSF 5'-flanking DNA sequences active in HTLV-I-infected T cells. The regulatory activity of the 5' deletion mutants was characterized in the HTLV-I-infected S-LB-I T-cell line. Consistent with their ability to produce GM-CSF constitutively, S-LB-I cells transfected with the +pCSFp1 construct generate CAT activity in the absence of stimulation (Fig. 2A; +pCSFp1 compared with −pCSFp1). Deletion of as many as 433 nucleotides from the 5' end of the 660-bp region (plasmid −193, which contains 193 nucleotides upstream from the GM-CSF initiation site) had no effect on the level of *cat* gene expression in stimulated or unstimulated S-LB-I cells (Fig. 2B). In contrast, removal of 14 additional base pairs from the 5' end (plasmid −179) resulted in a two- to threefold increase in CAT activity in unstimulated S-LB-I cells. Plasmid −106 (containing 106 nucleotides upstream of the GM-CSF cap site) behaved identically to plasmid −179, but deletion of an additional 38 nucleotides (plasmid −68) returned the level of CAT activity in the unstimulated cells back to that seen with +pCSFp1 or plasmid −193 (Fig. 2B).

These results suggest that a negative regulatory sequence is located between base pairs −193 and −179. Furthermore, sequences located between −106 and −68 contain positive regulatory activity in unstimulated S-LB-I cells. Neither of these regions, −193 to −179 nor −106 to −68, is required to demonstrate an increase in CAT activity after PHA-PMA stimulation, since plasmid −53 had the same activity as +pCSFp1 in S-LB-I cells.

The increase in CAT activity seen after activation of S-LB-I cells was specific. BUG-CAT- and −pCSFp1-directed CAT activity did not rise after stimulation. In addition, although pSV-CAT contains a PMA-responsive element (13), the CAT activity generated by pSV-CAT did not increase after stimulation of S-LB-I cells (Fig. 2A). (It is possible that the PMA-responsive element is already fully activated in unstimulated S-LB-I cells.)

In each experiment, the increase (three- to fourfold) in CAT activity after T-cell stimulation was identical to the increase in GM-CSF protein in the transfected-cell-conditioned medium (e.g., from 10 to 40 ng/ml), demonstrating that the changes in CAT activity reflect changes in GM-CSF protein level and thus correlate with the physiologic events.

DNase I footprinting studies. Having demonstrated positive and negative regulatory regions in the 5' flanking sequences of the GM-CSF gene, we performed DNase I footprinting experiments to determine whether cellular proteins bind to these regulatory regions. Using crude nuclear extracts from unstimulated MLA144 and S-LB-I cells, we observed a DNase I footprint about 34 bp long, from base pair −57 to base pair −24 (Fig. 3). This footprint is located upstream of the GM-CSF cap site and contains the first few base pairs of the TATTAA homology at its 3' end. Unstimulated MLA144 and S-LB-I crude nuclear extracts gave identical footprints when either the coding (Fig. 3) or the noncoding strand (not shown) was used. No change was seen when extracts were prepared from stimulated S-LB-I cells, but when stimulated MLA144 cells were used as a source of crude nuclear extract, a smaller, 19-bp footprint was observed (from −52 to −34). Crude nuclear extracts prepared from stimulated and unstimulated MLA144 cells produce indistinguishable DNase I footprints when assayed on the U3 region of the HTLV-I long terminal repeat (unpublished data), demonstrating that the change observed in the DNase I footprint of the GM-CSF promoter region is specific and is a result of PHA and PMA treatment of the cells.

The region protected from DNase I digestion is located within the region containing GM-CSF promoter activity. Within the 34- and 19-bp footprints is the repeated nucleotide

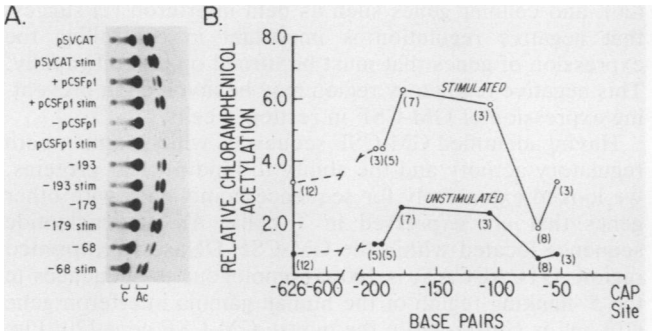


FIG. 2. Activity of GM-CSF-CAT constructions in the HTLV-I-infected S-LB-I cell line. (A) Assay of CAT activity generated by the deletion mutant plasmids in unstimulated and stimulated (stim) S-LB-I cells. Migration of acetylated chloramphenicol was from left to right. (B) Graph plotting GM-CSF promoter activity in unstimulated and stimulated cells versus the size of the recombinant plasmid. The relative chloramphenicol acetylation generated by each plasmid was determined by averaging 3 to 12 separate experiments (the exact numbers are shown in parentheses).

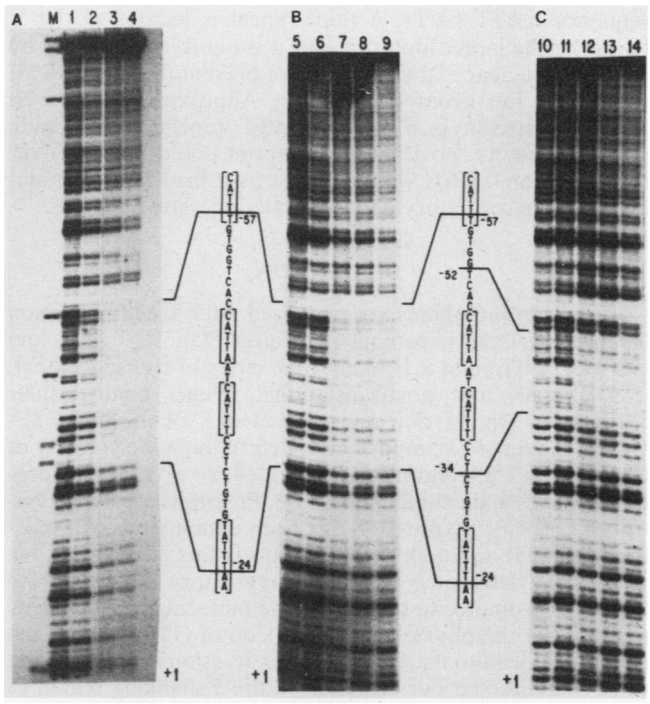


FIG. 3. DNase I footprinting of GM-CSF upstream sequences. The experiment was performed as described in Materials and Methods. Footprinting of the coding strand is shown. Maxam-Gilbert sequencing reactions were performing (data not shown) and used to identify particular bases in the DNase I footprint. (A) Binding reactions with S-LB-I crude nuclear extracts and 10 ng of DNase I. Lanes: M, size marker—*Sau*3A-digested pBR322; 1, no extract; 2, 25 μg of extract; 3, 100 μg of extract; 4, 200 μg of extract. (B) Binding reactions with MLA144 (unstimulated) crude nuclear extracts. Lanes: 5 and 6, no extract; 7, 8, and 9, 200 μg of extract and 5, 10, and 20 ng of DNase I, respectively. (C) Binding reactions with MLA144 (stimulated) crude nuclear extracts. Lanes: 10 and 11, no extract; 12, 13, and 14, 200 μg of extract and 5, 10, and 20 ng of DNase 1, respectively.

sequence CATT (A/T). A third repeat is located at the 5′ border of the larger footprint, but it is not fully protected by the crude nuclear extracts and is not present in plasmid −53, which has full promoter activity. Approximately 110 bp further upstream is a fourth CATT repeat. With crude nuclear extracts, no DNase I footprint could be seen over this upstream CATT(A/T) repeat or over the region containing negative regulatory activity (data not shown).

DISCUSSION

Various lymphokines are produced after T-cell activation (such as IL-2 [21], gamma interferon [25], IL-3 [42], and GM-CSF [41]), and it is likely that series of transcriptional, translational, and posttranslational events control their expression. An AT-rich region located 3′ of the GM-CSF-coding sequences is important in determining the stability of GM-CSF mRNA and mRNA encoded by a variety of protooncogenes and cellular genes (29). Posttranscriptional control of GM-CSF expression has been demonstrated in macrophages (34) and may be mediated via this 3′ region.

We have attempted to identify regulatory regions in the GM-CSF promoter to determine the molecular mechanisms that control the physiologic expression of GM-CSF and the events that lead to its constitutive expression in transformed cells. We directed our attention to the 5′-flanking region of the GM-CSF gene, thereby eliminating any possible contribution of 3′ sequences to the regulation of GM-CSF mRNA levels, and discovered a complex pattern of regulatory activities.

Our results show that the GM-CSF sequences located between base pairs −53 and +37 are sufficient for inducible GM-CSF promoter activity in all three T-cell lines tested. These sequences are not functional in unstimulated or mitogen-stimulated B-cell lines, consistent with their apparent T-cell specificity. The recently described phorbolester-responsive element (TRE) (1, 15) is not contained in these sequences; thus, other sequences are responsible for the regulatory activity seen.

We have observed differences in the activities of our deletion mutants in the presence or absence of HTLV infection. Cross and co-workers have also identified specific regulatory regions in the IL-2 receptor alpha chain (IL-2R) gene that were active only in the presence of HTLV infection (3). The mechanism by which HTLV infection activates cellular genes such as IL-2 receptor alpha or GM-CSF is unknown, but several investigators have demonstrated a direct effect of HTLV $p40^{x^I}$ on IL-2 receptor and promoter sequences (14, 16). We were unable to demonstrate an effect of the HTLV *trans*-activating proteins ($p40^{x^I}$ and $p37^{x^{II}}$) on GM-CSF 5′ regulatory sequences (data not shown) and therefore cannot attribute constitutive production of GM-CSF to direct activation of GM-CSF promoter sequences by pX proteins.

DNase I footprinting experiments with crude nuclear extracts from S-LB-I cells demonstrated the binding of nuclear protein(s) to DNA sequences contained within the GM-CSF promoter region. The 34-bp footprint (from −57 to −24) contains a repeated nucleotide sequence, CATT(A/T), which could serve as a recognition sequence for one or more cellular transcription factors. A third CATT repeat, located just upstream of the human GM-CSF promoter region and the DNase I footprint, is not present in the 5′-flanking region of the mouse GM-CSF gene (33), and our data showed that this third CATT repeat is not required for the promoter activity of the human GM-CSF gene in T cells. Although several transcription factors have been identified, no specific factor has been reported that recognizes the sequences CATT(A/T) or any other sequence contained within the DNase I footprint region.

The smaller DNase I footprint observed with crude nuclear extracts from stimulated MLA144 cells may reflect conformational changes in DNA-binding proteins resulting from cellular activation, or it may reflect a loss of protein binding upon induction. We observed a small but consistent increase in CAT activity in Jurkat and MLA144 cells upon deletion of the sequences between −68 and −53 (Fig. 1), so these sequences may be capable of binding a repressor-type protein in the uninduced state. It is also possible that different proteins bind to the DNA in the stimulated and unstimulated states. The fact that no change in this DNase I footprint was observed when stimulated S-LB-I cell extracts were used may reflect differences in the amounts or DNA-binding affinities of factors from S-LB-I versus MLA144 cell extracts, or it may be due to interactions with virally encoded proteins.

Located upstream of base pair −53 are additional regions that contain regulatory activity. Our studies with the HTLV-I-infected S-LB-I cell line suggested the presence of a negative regulatory region located between −193 and −179 and a positive regulatory region located between −106 and −68 (Fig. 4). The promoter activities of plasmids −68 and −53 demonstrate that these regions are not required for increasing CAT expression after T-cell stimulation. Studies with Jurkat cells also suggest the presence of a negative regulatory region (located between −623 and −193) which may be involved in preventing constitutive gene expression in unstimulated cells.

The data available suggest that GM-CSF acts as a local mediator in stimulating hematopoiesis and enhancing the inflammatory response. Local production of GM-CSF at a site of inflammation would direct phagocytes to the site, enhance their biological activities, and prevent their migration. An uncontrolled inflammatory response would lead to massive self-destruction of adjacent normal tissue, so production of GM-CSF must be tightly controlled to allow a rapid response to infections or other agents but also to limit the extent of that response.

The demonstration of a negative regulatory region in the GM-CSF gene and the demonstration of functionally similar elements in protooncogenes, such as c-*fos* (27) and c-*myc* (26), and cellular genes such as beta interferon (7) suggest that negative regulation is important in controlling the expression of genes that must be turned on and off rapidly. This negative regulatory region may be involved in preventing expression of GM-CSF in resting T cells.

Having identified GM-CSF sequences which contain both regulatory activity and the ability to bind nuclear proteins, we looked extensively for sequence homologies with other genes that are expressed in T cells. An octanucleotide sequence located within the GM-CSF DNase I footprinted region, GTCACCAT, is 100% homologous to sequences in the 5′-flanking region of the human gamma interferon gene (10) and is conserved in the mouse GM-CSF gene (20; Fig. 4B). Thus, it may play a role in controlling the expression of the lymphokines GM-CSF and gamma interferon. Several homologies can also be identified among sequences in the GM-CSF regulatory region and sequences within the HTLV-II (30) and the human immunodeficiency virus long terminal repeats (24; Fig. 4B). Seven nucleotides contained within the GM-CSF DNase I footprint are identical to sequences located just 3′ of the HTLV-II long terminal repeat initiation

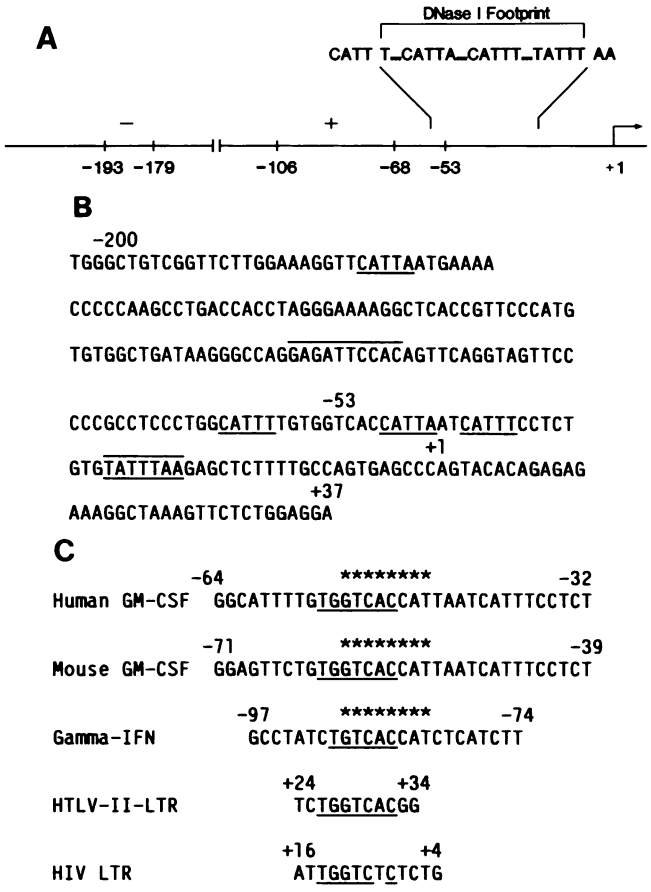


FIG. 4. (A) Localization of positive and negative regulatory sequences active in S-LB-I cells. The regions containing positive regulatory activity (−106 to −68), negative regulatory activity (−193 to −179), and inducible promoter activity (−53 to +37) are shown. The location of the DNase I footprint in S-LB-I cells is shown on the location of the CATT repeats, and the TATTAA homology is shown. (B) Nucleotide sequence of 5′-flanking region of the human GM-CSF gene. The location of the TATA homology (indicated by ——), the GM-CSF initiation site (+1), the CATT repeats (underlined), the lymphokine consensus sequences (indicated by a line over the nucleotides), and the GM-CSF promoter sequences (−53 to +37 are shown. (C) Sequence homologies. Abbreviations: IFN, interferon; LTR, long terminal repeat; HIV, human immunodeficiency virus.

site and 86% homologous with sequences present in the reverse orientation in a putative metal-binding finger contained within the R region of the human immunodeficiency virus long terminal repeat (23).

The decanucleotide lymphokine consensus sequence GPuGPuTTPyCAPy (33), found in the 5′-flanking sequences of the murine and human IL-2, IL-3, and GM-CSF genes, is repeated twice within the 660-bp fragment of the human GM-CSF sequences (Fig. 4). Our deletion mutant studies suggest that these repeats are not necessary for GM-CSF promoter activity. We have postulated the presence of a second positive regulatory region located between base pairs −106 and −68, which may be involved in the increased basal activity seen when upstream negative regulatory sequences are deleted in S-LB-I cells. The decanucleotide lymphokine consensus sequence is contained within this region (base pairs −100 to −91), but at the present time we cannot ascribe a regulatory function to it.

In summary, we observed a complex arrangement of regulatory elements located 5′ of the GM-CSF-coding sequences involving positive and negative regulatory activities. This arrangement would provide for tight regulation of GM-CSF expression, allowing strict control of hematopoiesis and the inflammatory response.

ACKNOWLEDGMENTS

We thank Alan Cann, Irvin Chen, and David Golde for helpful comments; Jeff Chan, Shirley Quan, and Tina Rhoades for technical assistance; and Wendy Aft for preparation of the manuscript.

This work was supported by Public Health Services grants CA23175, CA40163, CA32737, CA01811 (S.D.N.), and CA01113 (W.W.); American Cancer Society grant JFRA134 (J.C.G.); and grants from the Cancer Research Coordinating Committee (S.D.N.) and the California Institute of Cancer Research (W.W.).

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