MOLECULAR AND CELLULAR BIOLOGY, Nov. 1990, p. 6084–6088
0270-7306/90/116084-05$02.00/0

Vol. 10, No. 11

The Repeated Sequence CATT(A/T) Is Required for Granulocyte-Macrophage Colony-Stimulating Factor Promoter Activity

S. NIMER,[1]* J. FRASER,[1] J. RICHARDS,[1] M. LYNCH,[1] AND J. GASSON[1,2]

Department of Medicine, Division of Hematology-Oncology,[1] and Department of Biological Chemistry,[2] University of California, Los Angeles, School of Medicine and Jonsson Comprehensive Cancer Center, Los Angeles, California 90024-1678

Received 15 May 1990/Accepted 13 August 1990

The hematopoietic growth factor GM-CSF (granulocyte-macrophage colony-stimulating factor) is expressed by activated but not resting T lymphocytes. Previously, we localized GM-CSF-inducible promoter activity to a 90-bp region of GM-CSF 5′-flanking sequences extending from bp −53 to +37. To more precisely identify the GM-CSF DNA sequences required for inducible promoter activity in T lymphocytes, we have performed mutagenesis within a region of GM-CSF 5′-flanking sequences (bp −57 to −24) that contains the repeated sequence CATT(A/T). Mutations that do not alter the repeated CATT(A/T) sequence do not eliminate inducible promoter activity, whereas mutation or deletion of either of the CATT(A/T) repeats eliminates all inducible promoter activity in T-cell lines and in primary human T lymphocytes. Thus, both copies of the direct repeat CATT(A/T) are required for mitogen-inducible expression of GM-CSF in T cells.

GM-CSF (granulocyte-macrophage colony-stimulating factor) is a multilineage growth factor which stimulates the growth of early hematopoietic progenitors and also enhances the functional capabilities of mature myeloid cells (4, 5, 9). GM-CSF mRNA does not accumulate in normal unstimulated cells, but activation of T lymphocytes by antigen, mitogen, or lectin induces the expression of GM-CSF (3, 18). To understand the molecular mechanisms controlling the physiologic production of hematopoietic growth factors and to examine the coordinate expression of lymphokines, we have been analyzing regulatory sequences in the GM-CSF 5′-flanking region. We previously identified a region (bp −53 to +37) that contained mitogen-inducible promoter activity in a variety of T-cell lines and also bound nuclear proteins from crude T-lymphocyte nuclear extracts in DNase I footprint assays (13). Contained within these 90 bp is a 19-bp region of the GM-CSF gene (from −52 to −34) that bound nuclear proteins contained in crude nuclear extracts from the human T cell leukemia virus type I-infected S-LB-1 and the gibbon ape MLA 144 T-cell lines (see Fig. 1). The DNA sequences in this region contained the repeated sequence CATT(A/T), which we postulated could serve as the recognition sequence for a cellular transcription factor (13).

For our present studies, we have concentrated on the CATT(A/T) sequence which is repeated three times in the region from bp −62 to −37 [for ease in nomenclature, these repeats are numbered CATT −1, CATT −2, and CATT −3, moving upstream from the GM-CSF cap site (Fig. 1); a fourth CATT(A/T) repeat (CATT −4) is located at bp −175]. To identify the physiologically relevant GM-CSF promoter sequences that confer responsiveness to mitogenic signals in the T cell, we first created a series of internal deletion mutations lacking a variable number of nucleotides from the footprint region. These deletion mutations were constructed utilizing a unique *Bst*EII site within the stimulated footprint region (position −53 relative to the GM-CSF cap site) and variable limited BAL 31 exonuclease digestion. Mutant constructs were sequenced by the dideoxy sequencing method of Sanger to determine the exact sequences deleted. Internal deletion mutant GM-CSF promoter-chloramphenicol acetyltransferase (CAT) reporter gene constructs were electroporated into the MLA 144 T-cell line as previously described (11) and into primary human T lymphocytes (2). For MLA 144 cells, the transiently transfected cells were either stimulated with 1% phytohemagglutinin (PHA) and 12-*O*-tetradecanoylphorbol-13-acetate (20 ng/ml) or left unstimulated for 16 h before the CAT activity was assayed by standard methods (6). For primary human T-cell electroporations, primary human T cells were isolated from Leukopaks (purchased from the American Red Cross) by Ficoll-Hypaque separation followed by two-step adherence to plastic to remove the majority of the monocytes present. The cells were grown in the presence of PHA (1%) for 48 h prior to electroporation, and following the electroporations, the cells were stimulated for an additional 24 h with PHA (1%) before being harvested for assay of CAT activity.

This approach allowed us to measure inducible promoter activity in T-cell lines and to characterize GM-CSF promoter activity in primary human peripheral blood T cells. As shown in Fig. 2, elimination of 14 bp located just upstream of CATT −2 (+pCSFp1Δ−62/−49) did not diminish inducible promoter activity in MLA 144 cells compared with the wild-type +pCSFp1 construct, nor did it reduce promoter activity in PHA-stimulated primary human T cells. However, deletion of 22 bp, including 19 bp from within the DNase I footprint region (+pCSFp1Δ−60/−39), eliminated all promoter activity in both MLA 144 cells and primary T cells. This construct no longer contains the CATT −2 and CATT −1 repeats, although the remainder of the GM-CSF 5′-flanking sequences (~607 bp) remains intact. A similar plasmid, +pCSFp1Δ−61/−38, which lacks 24 bp (20 bp within the DNase I footprint region), also had no inducible regulatory activity, nor did plasmid +pCSFp1Δ−59/−42, which has an 18-bp internal deletion. The deletion in plasmid +pCSFp1Δ−59/−42 eliminated the CATT −2 repeat without disrupting the CATT −1 repeat; its lack of activity demonstrates that the presence of the CATT −1 repeat itself is not sufficient for inducible promoter activity. Eliminating the 5 bp immediately upstream of the CATT −2 repeat,

* Corresponding author.

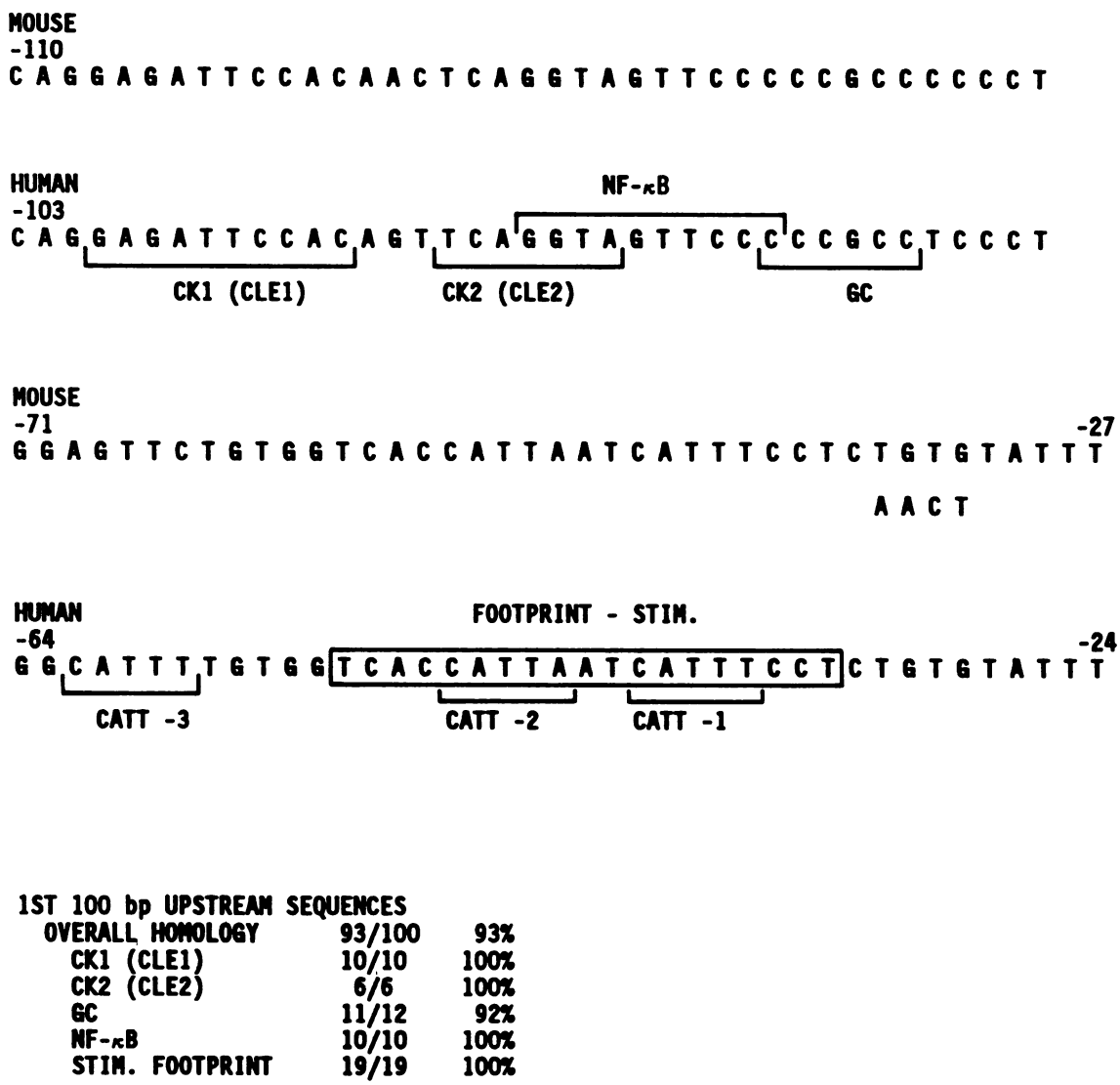
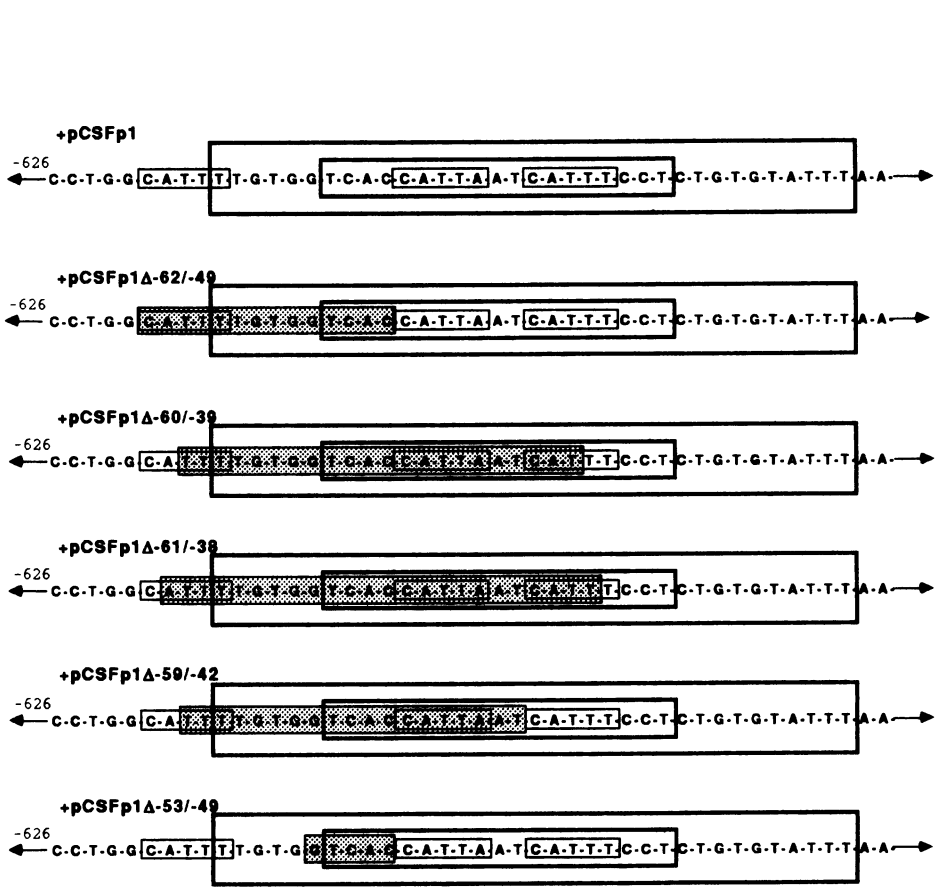


FIG. 1. Comparison of the human and murine GM-CSF 5'-flanking regions. DNA sequences are shown for the human (extending from bp −24 to −103 relative to the GM-CSF cap site) and the murine (extending from bp −27 to −110) upstream regions. The sequences in the human GM-CSF promoter protected from DNase I digestion by crude nuclear extracts from stimulated MLA 144 cells are outlined. The locations of the CATT −3, CATT −2, and CATT −1 repeats are indicated. The locations of regions identified by others as containing potential regulatory elements are indicated. CK1 (15) and CLE1 (10) are different names given to the same region, as are CK2 (15) and CLE2 (10). CK1 (CLE1) is identical to the region we have previously referred to as LC2 (13). The GC-rich region and the region capable of binding NF-κB are also indicated. Stim., Stimulated.

construct +pCSFp1Δ−53/−49, left 40 to 55% of the regulatory activity intact; as discussed below, the decrease in promoter activity of this construct may be the result of bringing a negative regulatory element closer to the GM-CSF-inducible promoter sequences.

The activities of these deletion mutant constructs in stimulated T cells directly parallel their inducible activities in the T-cell lines. Taken together, these results show that constructs containing deletions that eliminate either the CATT −2 repeat or both of the downstream CATT(A/T) repeats lack promoter activity and that deletions that remove sequences contained within the DNase I footprint but leave the CATT(A/T) repeats intact have full promoter activity.

To more precisely identify the GM-CSF regulatory sequences in the region −52 to −34 and to eliminate possible effects of altered spacing between regulatory elements, we constructed oligonucleotide-directed site-specific mutations of either one or both of the CATT −2 and CATT −1 repeats. Synthetic oligonucleotides (60-mers) containing the base pair substitutions indicated in Fig. 3 were gel purified and hybridized to a single-stranded wild-type GM-CSF template (the *Sac*I fragment from +pCSFp1 [bp −458 to −16]) in m13mp18. A mutant double-stranded plasmid (RF DNA) was generated (with the Amersham mutagenesis kit), and the mutated *Sac*I fragment was isolated and used to recreate the site-specific mutated form of the +pCSFp1 plasmid. Nucleotide substitutions within both the CATT −2 and CATT −1 repeats eliminated inducible promoter activity in primary human T lymphocytes or stimulated MLA cells (Fig. 3, +pCSFp1ΔCATT −1, −2). Constructs containing a mutation of a single CATT(A/T) repeat (either a 2-bp substitution in the CATT −2 repeat or a 3-bp substitution in the CATT



	RELATIVE CAT ACTIVITY IN MLA 144 CELLS			CAT ACTIVITY IN STIMULATED PRIMARY T CELLS
	UNSTIMULATED	STIMULATED	FOLD INDUCTION	
+pCSFp1	1.0*	36.2	36.2x (N=13)	3.8 (N=7)
+pCSFp1Δ-62/-49	1.8	84.1	46.7x (N=6)	6.8 (N=7)
+pCSFp1Δ-60/-39	0.6	0.7	1.2x (N=10)	1.0* (N=7)
+pCSFp1Δ-61/-38	0.6	1.1	1.8x (N=2)	0.8 (N=3)
+pCSFp1Δ-59/-42	0.5	1.2	2.4x (N=8)	1.8 (N=3)
+pCSFp1Δ-53/-40	0.6	8.9	14.8x (N=8)	2.1 (N=4)

*Defined as 1.0 (Actual % Acetylation = 0.4%)

FIG. 2. Regulatory activity of GM-CSF promoter deletion mutations. All constructs extend from bp +37 (relative to the GM-CSF cap site) to bp −626, though only the sequences immediately upstream of the TATA homology are depicted. The wild-type GM-CSF CAT construct (+pCSFp1) is diagrammed on the top line; the DNA sequences in the wild-type GM-CSF promoter that are protected from DNase I digestion by crude nuclear extracts from either unstimulated MLA 144 cells (the boldface larger, outer box) or from stimulated MLA 144 cells (the boldface smaller, inner box) are indicated. The 5-bp repeated sequence CATT(A/T) is outlined by a thinner rectangular box. The base pairs deleted from the mutant constructs are shaded. The plasmids are named according to the locations of the base pairs deleted (e.g., plasmid +pCSFp1Δ−60/−39 lacks bp −60 to −39, inclusive). The amount of CAT activity generated by these constructs in MLA 144 cells (percent acetylation of the chloramphenicol substrate) is reported relative to the activity of the +pCSFp1 plasmid in unstimulated MLA 144 cells. The extent of induction was computed by dividing the relative CAT activity in stimulated cells by the relative CAT activity in unstimulated cells. The CAT activity in PHA-stimulated primary T lymphocytes is reported relative to the activity of the plasmid +pCSFp1Δ−60/−39, which generates only background amounts of CAT activity in T-cell lines (6). The number (N) of experiments performed using each construct is indicated.

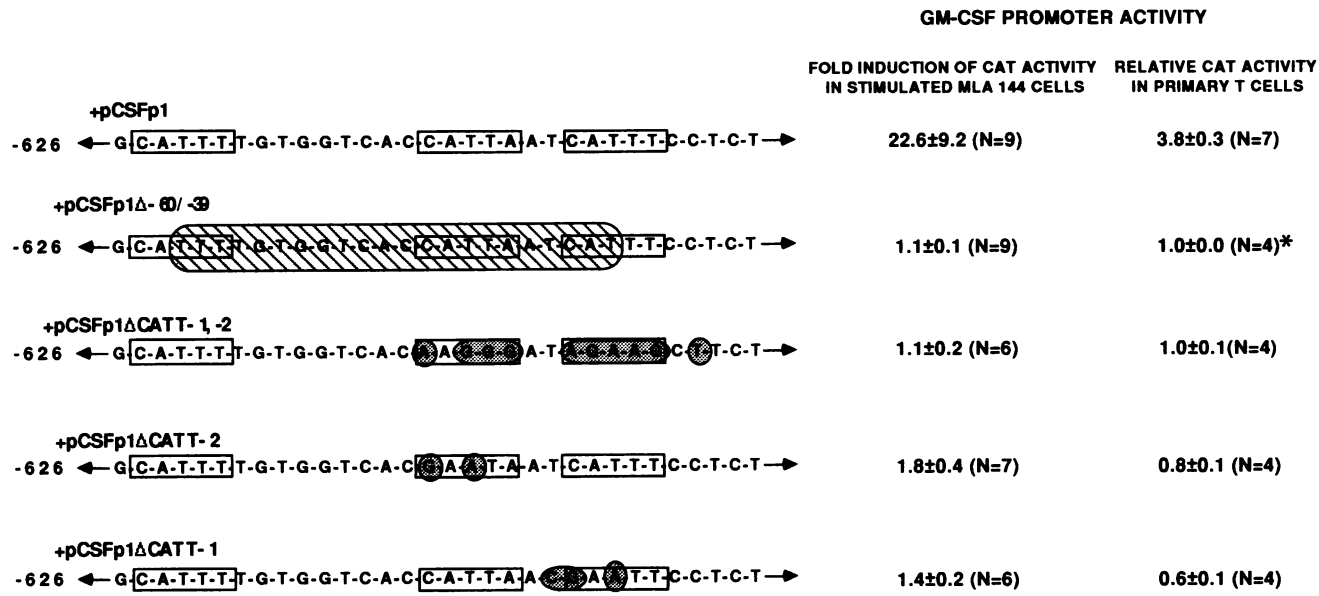


FIG. 3. Regulatory activity of oligonucleotide-directed site-specific mutations. The sequences of oligonucleotides used to create mutations are shown with substituted base pairs indicated by shaded regions. The wild-type sequence is shown on the top line. All constructs extend to bp −626 relative to the GM-CSF cap site. N, Number of experiments. Symbol: *, defined as 1.0 in each experiment.

−1 repeat) also manifested no inducible promoter activity in primary T cells or in the T-cell lines (Fig. 3, lines 3 and 4). On the basis of these data, we conclude that both downstream CATT(A/T) repeats are essential for GM-CSF-inducible promoter activity in T cells. Although we cannot identify precisely which nucleotides within the CATT(A/T) repeats are critical for recognition by transcription factors, we find that both of these repeats are required.

We examined the binding of nuclear factors from both fresh human primary T lymphocytes and T-cell lines to wild-type and mutated GM-CSF promoter sequences by using crude nuclear extracts. The mutations that eliminated promoter activity also disrupted the DNase I footprint pattern (unpublished data), but the changes observed were not as dramatic as the effects of these mutations on promoter activity. Studies using fast protein liquid chromatography-fractionated extracts are in progress and may prove to be more illuminating in attempts to correlate changes in DNA-protein interactions with changes in promoter activity.

A number of reports have identified other human or murine GM-CSF 5′ sequences (i.e., the lymphokine decanucleotide consensus sequence CK1, the CK2 and GC-rich sequences, and an NF-κB recognition sequence) capable of binding nuclear proteins from either T cells or non-T-cell lines (7, 10, 14–16) (Fig. 1). These sequences are located upstream of the GM-CSF sequences required for mitogen-inducible expression in T cells (8, 13) or for promoter activity in stimulated fibroblasts (12) or endothelial cells (8). These sequences may serve to modulate the activities of the GM-CSF promoter sequences we have identified in these experiments. None of these upstream sequences are required for inducible GM-CSF promoter activity (12, 13), and none are sufficient for transcription from these mutant GM-CSF CAT constructs that contain an intact TATA homology and transcriptional initiation site. The plasmid −53, which contains only the sequences between bp −53 and +37, is fully responsive to mitogen and to transactivation by the human T cell leukemia virus types I and II Tax proteins in T cells (11, 13). This construct also has basal promoter activity in fibroblasts that constitutively transcribe the GM-CSF gene (12).

The minimal GM-CSF promoter that we have identified, plasmid −53, does not contain the sequences capable of binding the nuclear factors NF-GMa and NF-GMb (15). Recently sequences capable of binding the nuclear factor NF-κB have been identified in the 5′-flanking region of the mouse (and human) GM-CSF genes (14). Previous deletion mutagenesis studies have demonstrated that this NF-κB sequence is not required for Tax responsiveness of the GM-CSF promoter (11), and our current studies show that deletions within the footprint region, which leave the NF-κB site intact, completely eliminate promoter function.

Our data also suggest that the region containing the CATT −3 repeat may function as a negative regulatory element in that both plasmid −53 and +pCSFp1Δ−62/−49 have increased constitutive promoter activity compared with +pCSFp1. In addition, we postulate that removal of the 5 bp from the +pCSFp1Δ−53/−49 construct (Fig. 2), which moves the CATT −3 repeat closer to the CATT −2 and CATT −1 repeats, may interfere with the appropriate recognition of the regulatory element (CATT −2 and CATT −1) by transcription factors required for promoter activity. In other studies, we have found that the region containing the CATT −3 repeat has potent negative regulatory activity in myeloid cells (J. K. Fraser et al., submitted for publication).

Having demonstrated the importance of the CATT(A/T) repeats in regulating GM-CSF expression, we examined the 5′-flanking region of other lymphokine genes to determine whether a similar motif was present. Interleukin-3 is a related hematopoietic growth factor, but the interleukin-3 gene, which is located only 9.0 kb from the GM-CSF gene on the long arm of chromosome 5 (19), does not contain similar CATT(A/T) repeats in the 800 bp of its 5′ upstream region. Of the other lymphokine genes, only the interleukin-5 gene, which like GM-CSF encodes a protein with potent effects on eosinophils and is also located on human chromosome 5q (17), has a homologous region in its upstream sequences (Fig. 4) (1). This sequence is preserved in both the human

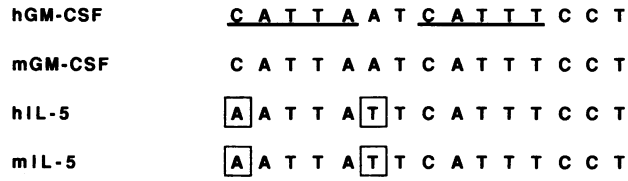


FIG. 4. Comparison of upstream sequences in the human GM-CSF promoter (hGM-CSF) protected from DNase I digestion with homologous sequences in the upstream regions of the human and murine interleukin-5 genes (hIL-5 and mIL-5, respectively) and the murine GM-CSF gene (mGM-CSF).

and mouse interleukin-5 genes, which may imply some role in regulating coordinate expression of these genes (1).

We thank Kenneth Hu, Chi Nguyen, and Missie Joe Gates for technical assistance and Linda Rodman for preparation of the manuscript.

This work was supported by Public Health Service grants CA 01811 (S.D.N.) and CA 32737 and CA 40163 (J.C.G.) from the National Institutes of Health, a California Institute for Cancer research grant and Leukemia Society of America Fellowship (J.K.F.), and grants from the Leukemia Research Foundation (S.D.N.) and Cancer Research Coordinating Committee (S.D.N.). J.C.G. is a Scholar of the Leukemia Society of America.

LITERATURE CITED

1. **Campbell, H. D., G. J. Sanderson, Y. Wang, Y. Hort, M. E. Martinson, W. Q. J. Tucker, A. Stellwagen, M. Strath, and I. G. Young.** 1988. Isolation, structure and expression of cDNA and genomic clones for murine eosinophil differentiation factor. Comparison with other eosinophilopoietic lymphokines and identity with interleukin-5. Eur. J. Biochem. **174**:345–352.
2. **Cann, A. J., Y. Koyanagi, and I. S. Y. Chen.** 1988. High efficiency transfection of primary human lymphocytes and studies of gene expression. Oncogene **3**:123–128.
3. **Chan, J. Y., D. J. Slamon, S. D. Nimer, D. W. Golde, and J. C. Gasson.** 1986. Regulation of expression of human granulocyte/macrophage colony-stimulating factor. Proc. Natl. Acad. Sci. USA **83**:8669–8673.
4. **Clark, S. C., and R. Kamen.** 1987. The human hematopoietic colony-stimulating factors. Science **236**:1229–1237.
5. **Golde, D. W., and J. C. Gasson.** 1988. Cytokines: myeloid growth factors, p. 253–261. *In* J. I. Gallin, I. M. Goldstein, and R. Snyderman (ed.), Inflammation: basic principles and clinical correlates. Raven Press Publishers, New York.
6. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
7. **James, R., and J. Kazenwadel.** 1989. T-cell nuclei contain a protein that binds upstream of the murine granulocyte-macrophage colony-stimulating factor gene. Proc. Natl. Acad. Sci. USA **86**:7392–7396.
8. **Kaushansky, K.** 1989. Control of granulocyte-macrophage colony-stimulating factor production in normal endothelial cells by positive and negative regulatory elements. J. Immunol. **143**: 2525–2529.
9. **Metcalf, D.** 1985. The granulocyte-macrophage colony-stimulating factors. Science **229**:16–22.
10. **Miyatake, S., M. Seiki, M. Yoshida, and K.-I. Arai.** 1988. T-cell activation signals and human T-cell leukemia virus type I-encoded $p40^x$ protein activate the mouse granulocyte-macrophage colony-stimulating factor gene through a common DNA element. Mol. Cell. Biol. **8**:5581–5587.
11. **Nimer, S. D., J. C. Gasson, K. Hu, I. Smalberg, J. L. Williams, I. S. Y. Chen, and J. D. Rosenblatt.** 1989. Activation of the GM-CSF promoter by HTLV-I and -II tax proteins. Oncogene **4**:671–676.
12. **Nimer, S. D., M. J. Gates, H. P. Koeffler, and J. C. Gasson.** 1989. Multiple mechanisms control the expression of granulocyte-macrophage colony-stimulating factor by human fibroblasts. J. Immunol. **143**:2374–2377.
13. **Nimer, S. D., E. A. Morita, M. J. Martis, W. Wachsman, and J. C. Gasson.** 1988. Characterization of the human granulocyte-macrophage colony-stimulating factor promoter region by genetic analysis: correlation with DNase I footprinting. Mol. Cell. Biol. **8**:1979–1984.
14. **Schreck, R., and P. A. Baeuerle.** 1990. NF-κB as inducible transcriptional activator of the granulocyte-macrophage colony-stimulating factor gene. Mol. Cell. Biol. **10**:1281–1286.
15. **Shannon, M. F., J. R. Gamble, Jr., and M. A. Vadas.** 1988. Nuclear proteins interacting with the promoter of the human granulocyte/macrophage colony-stimulating factor gene. Proc. Natl. Acad. Sci. USA **85**:674–678.
16. **Stanley, E. R., D. Metcalf, P. Sobieszczuk, N. M. Gough, and A. R. Dunn.** 1985. The structure and expression of the murine gene encoding granulocyte-macrophage colony stimulating factor: evidence for utilization of alternative promoters. EMBO J. **4**:2569–2573.
17. **Sutherland, G. R., E. Baker, D. F. Callen, H. D. Campbell, I. G. Young, C. J. Sanderson, O. M. Garson, A. F. Lopez, and M. A. Vadas.** 1988. Interleukin-5 is at 5q31 and is deleted in the 5q-syndrome. Blood **71**:1150–1152.
18. **Wong, G. G., J. S. Witek, P. A. Temple, K. M. Wilkens, A. C. Leary, D. P. Luxenburg, S. S. Jones, E. L. Brown, R. M. Kay, E. C. Orr, C. Shoemaker, D. W. Golde, R. J. Kaufman, R. M. Hewick, E. A. Wang, and S. C. Clark.** 1985. Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science **228**:810–815.
19. **Yang, Y.-C., S. Kovacic, R. Kriz, S. Wolf, S. C. Clark, T. E. Wellems, A. Nienhuis, and N. Epstein.** 1988. The human genes for GM-CSF and IL 3 are closely linked in tandem on chromosome 5. Blood **71**:958–961.