Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8669–8673, November 1986
Genetics

Regulation of expression of human granulocyte/macrophage colony-stimulating factor

(human T-cell leukemia virus/transcriptional regulation/chloramphenicol acetyltransferase/gene expression, cell type-specific)

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Communicated by Paul A. Marks, July 22, 1986

ABSTRACT **Colony-stimulating factors (CSFs) are glycoproteins that stimulate the growth of hematopoietic progenitors and enhance the functional activity of mature effector cells. Human granulocyte/macrophage colony-stimulating factor (GM-CSF) is a 22-kDa glycoprotein that stimulates the growth of myeloid and erythroid progenitors *in vitro* and increases the responsiveness of neutrophils, monocytes, and eosinophils to physiologic stimuli. Elucidation of the cell and tissue sources of CSFs, as well as study of their regulation of expression, is required to understand their role in physiologic and pathophysiologic states. An extensive survey of normal and neoplastic human tissues did not reveal constitutive production of detectable levels of GM-CSF mRNA in any of the 64 samples studied. Antigen- or lectin-activated T lymphocytes have been shown to produce GM-CSF; therefore, to elucidate the genetic sequences required, we constructed recombinant plasmids containing 5′ flanking DNA of the GM-CSF gene linked to the marker chloramphenicol acetyltransferase gene. The recombinant constructs were transfected into a human T-cell leukemia virus type I (HTLV)-infected T-lymphoblast cell line that can be stimulated to produce high levels of GM-CSF. We show here that the 5′ flanking sequences of the GM-CSF gene can direct increased expression of the chloramphenicol acetyltransferase gene in activated T-lymphoblast cells.**

Hematopoietic progenitor cells give rise to colonies of differentiated cells in semisolid medium in the presence of colony-stimulating factors (CSFs) (1). CSFs also affect the function of mature peripheral effector cells, and previous studies (2, 3) have implicated CSFs to be primary regulators of granulopoiesis. The biological and molecular properties of several human and murine CSFs have been characterized (reviewed in refs. 4 and 5). Human granulocyte/macrophage colony-stimulating factor (GM-CSF) is a 22-kDa glycoprotein originally purified from medium conditioned by the human T-cell leukemia virus type II (HTLV-II)-infected T-lymphoblast cell line Mo (6). cDNA clones encoding functional protein have been isolated (7). Both purified natural and biosynthetic (recombinant) GM-CSF stimulate the formation of granulocyte, macrophage, granulocyte/macrophage, and eosinophil colonies, as well as erythroid bursts from normal progenitors *in vitro* (8–10). Colony formation by the human myeloid leukemic cell lines HL-60 and KG-1 is also increased by GM-CSF (8). Like other CSFs, GM-CSF has direct actions on peripheral blood neutrophils; it enhances chemotaxis, phagocytosis, and superoxide production in response to physiologic stimuli such as *N*-formylmethionyl-leucylphenylalanine and the complement derivative C5a (6, 11–13). In addition, GM-CSF enhances antibody-mediated killing of *Schistosoma* larvae by human eosinophils *in vitro* (14). The gene encoding GM-CSF is 2.5 kilobases (kb) long, with three intervening sequences, and maps to the long arm of chromosome 5 (15, 16).

Previous work has shown that GM-CSF is a T-cell-derived growth factor. Phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes produce GM-CSF, as do all HTLV-I- or HTLV-II-infected T-cell lines tested (7, 17). The basal level of GM-CSF production in the HTLV-infected cell lines can be increased up to 10-fold after PHA and phorbol 12-myristate 13-acetate (PMA) treatment (7).

In this study, we have investigated the cellular sources of GM-CSF mRNA and protein. We have used a recombinant construction to identify a region of the GM-CSF gene involved in regulating expression in stimulated T-cell lines.

MATERIALS AND METHODS

GM-CSF Radioimmunoassay. Antisera were raised against GM-CSF by injection of rabbits with purified biosynthetic protein (≈5 μg) in complete Freund's adjuvant at multiple intradermal sites. Rabbits were given booster injections at monthly intervals with the same protein in incomplete Freund's adjuvant and were bled from the ear vein 10 days after each injection. Antisera were titrated using 25,000–30,000 cpm of ^{125}I-labeled (chloramine-T method) GM-CSF per tube, and antigen–antibody complexes were precipitated with fixed *Staphylococcus aureus* cells. The dilution of antiserum that precipitated 10,000–15,000 cpm of ^{125}I-labeled GM-CSF was used in the radioimmunoassay. The GM-CSF standard was purified biosynthetic human GM-CSF produced by transfection of monkey COS cells, as described (6, 7). The concentration of the standard was estimated by amino acid analysis. Multiple dilutions of samples were assayed in 0.2 ml (final volume) of Dulbecco's phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) containing 0.2% Nonidet P-40, 0.2 mg of bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride, 25,000–30,000 cpm of ^{125}I-labeled GM-CSF, the appropriate dilution of antiserum (1:2500 in Table 1), and carrier rabbit immunoglobulin. After overnight incubation at 4°C, goat anti-rabbit serum was added and incubation was continued for an additional 3 hr. After centrifugation, pelleted radioactivity was measured in a gamma counter, and dilutions of the sample were compared to dilutions of the standard. Previous work (unpublished) has shown that only medium conditioned by cells known to make GM-CSF (as determined by bioassay and RNA hybridization) contains GM-CSF detectable by this radioimmunoassay and that the assay does not detect macrophage CSF or granulocyte CSF.

Construction of Plasmids. Plasmid constructs were prepared by standard procedures. The plasmid pCHPO.8 was derived from the GM-CSF genomic clone pCH5.2 (15).

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Abbreviations: CSF, colony-stimulating factor; GM-CSF, granulocyte/macrophage CSF; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; HTLV, human T-cell leukemia virus; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; IL-1, IL-2, and IL-3, interleukins 1–3; bp, base pair(s).

pCH5.2 was digested with *Hin*dIII and *Pvu* II, generating a fragment of ≈800 base pairs (bp) containing the first exon and its flanking sequences. The fragment was then ligated into the polylinker of pUC18 (18) at the *Hin*dIII and *Hin*cII sites, creating pCHPO.8. Fragments containing only upstream sequences from the translational initiation codon of GM-CSF were generated by BAL-31 nuclease digestion of pCHPO.8 at the *Bam*HI site, followed by *Hin*dIII digestion. pCP6 was created by insertion of the fragments into pUC18 at the *Hin*dIII and *Hin*cII sites. pCP6 contains an insert of ≈660 bp, upstream from the initiator ATG of GM-CSF. The *Hin*dIII fragment containing the simian virus 40 (SV40) early promoter and the nonfunctional immunoglobulin sequence of BUG-CAT (kindly provided by J. Banerji, Harvard University Medical School) was replaced with the *Hin*dIII–*Bam*HI insert of pCP6 in both orientations. Ligation of the *Bam*HI and *Hin*dIII sites was achieved by filling in the single-stranded DNA, using Klenow fragment of DNA polymerase I, followed by blunt-end ligations. The exact endpoint of the 5′ GM-CSF fragment in +pCSFPI, as determined by Maxam–Gilbert sequencing (19), is 37 bp downstream from the cap site.

Transfection and CAT Assays. Cells were fed the day before transfection and washed with serum-free Iscove's modified Dulbecco's medium (IMDM) prior to the procedure. For each transfection, 10^7 cells were incubated in 50-ml Falcon tubes containing 10 ml of serum-free IMDM with DEAE-dextran (0.25 mg/ml), 0.1 M Tris/HCl (pH 7.4), and DNA (1 μg/ml) for 1.5 hr at 37°C. The cells were then washed with serum-free IMDM and incubated in IMDM supplemented with 5% fetal bovine serum and 0.1 mM chloroquin for 2.5 hr at 37°C. The cells were washed again and transferred to Falcon tissue culture plates containing 10 ml of IMDM with 20% fetal bovine serum. Twenty-four hours later, S-LB-I cells (HTLV-I-transformed normal T cells; ref. 17) were stimulated with PMA (Pharmacia) alone or PMA and either PHA (reagent grade, Wellcome) or monoclonal antibody OKT3 (Ortho Diagnostics). The Epstein–Barr virus-infected lymphoblastoid cell line PR (20) was similarly transfected and later stimulated with either bacterial lipopolysaccharide, pokeweed mitogen, or PMA. Twenty hours after stimulation, cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity, as described (21). CAT activity was determined by the acetylation of ^{14}C-labeled chloramphenicol assayed by thin-layer chromatography and autoradiography.

Nuclease S1 Analysis. The transcriptional start site of GM-CSF was determined by nuclease S1 analysis (22, 23). Probes were end-labeled by bacteriophage T4 polynucleotide kinase to a specific activity of about 10^7 cpm/μg of DNA. Approximately 0.02 μg of the probe was hybridized to 20 μg of total cellular RNA in 20 μl of 80% deionized formamide plus 5 μl of 2 M NaCl/200 mM Pipes, pH 6.4/5 mM EDTA, pH 7.0. Hybridization was allowed to proceed at 53°C for 12 hr. After hybridization, the samples were digested with 40 units of nuclease S1 in 250 μl of S1 digestion buffer for 1 hr at 37°C. S1-protected DNA was ethanol-precipitated, electrophoresed in an 8 M urea/10% acrylamide gel, and autoradiographed.

RESULTS

Cell and Tissue Sources of GM-CSF. GM-CSF mRNA is present in activated but not resting T lymphocytes (7). To determine whether any human tissues constitutively produce GM-CSF, poly(A)$^+$ RNA was prepared as described (24) from various fresh neoplasms and the surrounding normal tissue and screened by dot blot hybridization analysis with the full-length GM-CSF cDNA as a probe. No GM-CSF message was detected in 57 samples including liver, colon, esophagus, rectum, stomach, breast, lung, skin, seminona, neuroblastoma, melanoma, renal, and ovarian carcinomas and surrounding normal tissue. In addition, 7 leukemic cell samples were tested, including acute myelogenous leukemia, acute lymphoblastic leukemia, and acute myelomonocytic leukemia (data not shown). The limit of detection of this method is 40 pg of a specific transcript in 4 μg of poly(A)$^+$ RNA (24). The RNA samples were fractionated by electrophoresis in agarose gels in order to substantiate the integrity of the RNAs, and the dot blots were hybridized with an erythroid-potentiating activity (EPA) probe. The EPA probe has been found to be expressed by most human cells (unpublished data), and all the samples showed hybridization to the probe, thus confirming their integrity.

Activated T Cells Produce Increased Levels of GM-CSF. Previous studies (7), using blot hybridization analysis of electrophoretically fractionated RNA, have shown that HTLV-infected T-lymphoblast cell lines increased the expression of GM-CSF mRNA when treated with PHA/PMA (7). Nuclease S1 analysis of RNA from the S-LB-I (HTLV-I-infected) cell line was performed, using a fragment derived from the previously isolated GM-CSF genomic clone (15). The probe was the *Hin*fI–*Dde* I fragment from plasmid pCHPO.8, encompassing nucleotides −70 to 196 and containing the first exon and its 5′ flanking sequences. The 266-nucleotide fragment was 5′-labeled at the *Dde* I site. Two bands at ≈165 bp were protected, and the level of GM-CSF transcript increased ≈10-fold after 20 hr of treatment of S-LB-I cells with 0.5% PHA and 10 ng of PMA per ml (Fig. 1). To determine whether the levels of GM-CSF mRNA are reflected in increased protein, we developed a radioimmunoassay to estimate the concentration of GM-CSF protein in conditioned media from stimulated and unstimulated S-LB-I cells. In four separate experiments, the radioimmunoassay detected a 3- to 7-fold increase in GM-CSF

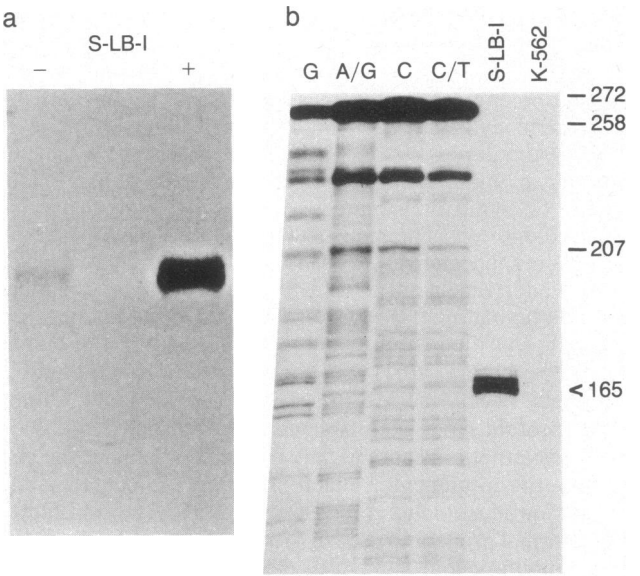

FIG. 1. (*a*) GM-CSF RNA is increased after stimulation with PHA and PMA. Nuclease S1 analysis was performed using total RNA from nonstimulated (−) or stimulated (+) S-LB-I cells. Unstimulated S-LB-I cells show constitutive production of GM-CSF RNA, and the level is increased ≈10-fold after 20 hr of stimulation with 0.5% PHA and 10 ng of PMA per ml. (*b*) Nuclease S1 analysis of the transcriptional start site of GM-CSF mRNA from stimulated S-LB-I cells. Lanes 1–4 (from left to right): Maxam–Gilbert sequencing reactions. Lane 5: S1 analysis using total RNA from S-LB-I cells 20 hr after PHA/PMA stimulation. Lane 6: S1 analysis using total RNA from K-562 cells, which do not produce GM-CSF. Molecular size (length in bp) markers from *Sau*3A-digested pBR322 are indicated at right.

protein (Fig. 2 and Table 1) after 20 hr of stimulation with 0.5% PHA and 10 ng of PMA per ml.

Activated T Cells Express the +pCSFPI Construct. Our studies did not reveal the constitutive expression of GM-CSF mRNA in normal tissues or cells, suggesting that antigen-activated T lymphocytes are one important physiological source of GM-CSF. In addition, GM-CSF mRNA cannot be detected in resting T cells; similarly, the constitutive level of expression in HTLV-infected T-lymphoblast cell lines is increased by treatment with PHA/PMA (ref. 7 and Fig. 1). To define the nucleotide sequences required in cis for expression of GM-CSF in activated T cells, we prepared recombinant constructs of the GM-CSF gene linked to the CAT gene and used the S-LB-I cell line as a model for activated T lymphocytes.

There is much evidence demonstrating the importance of sequences upstream from the cap site in the regulation of transcription (25). Thus, to include the cap site in the construction of +pCSFPI and −pCSFPI, DNA sequencing [Maxam and Gilbert method (19)] was performed using the same *Hin*fI–*Dde* I probe and analyzed simultaneously with the S1 analysis. The sequencing "ladder" shows the initiation site at nucleotides C and G at positions 1 and 3, respectively (Fig. 1*b*).

A 660-nucleotide fragment 5′ from the initiation codon of the GM-CSF gene and including the cap site (see above) was subcloned into the BUG-CAT plasmid, replacing its SV40 early promoter and nonfunctional immunoglobulin sequence (Fig. 3). The recombinant construct in the positive orientation (+pCSFPI) and that in the reverse orientation (−pCSFPI) were transfected into S-LB-I cells using a DEAE-dextran transfection procedure (see *Materials and Methods*). The cells were harvested with and without stimulation with PHA/PMA and assayed for CAT enzyme activity. +pCSFPI-transfected cells showed increased CAT levels when stimulated with 0.5% or 1.0% PHA and 10 ng of PMA per ml (Fig. 4). +pCSFPI-transfected cells also showed an increase in CAT activity when stimulated with PMA alone (data not shown). Nonstimulated +pCSFPI-transfected cells showed CAT activity levels similar to stimulated and non-

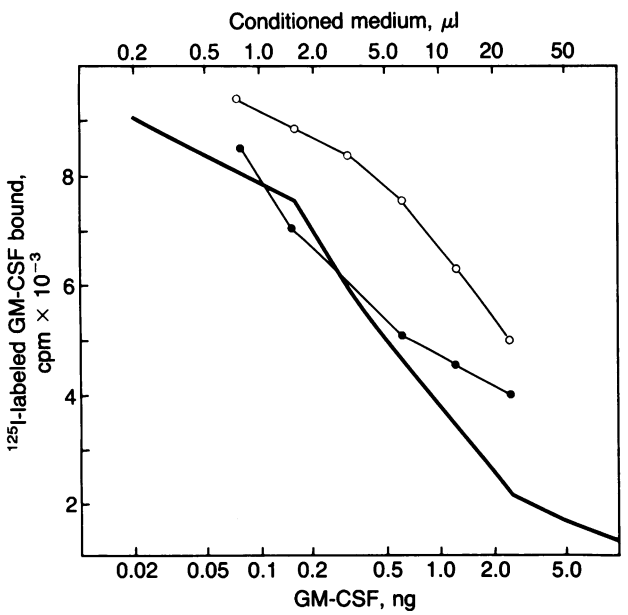

FIG. 2. GM-CSF production by S-LB-I cells increases after stimulation with PHA and PMA. Rabbit antiserum directed against GM-CSF was used in a radioimmunoassay to determine GM-CSF levels in medium conditioned by nonstimulated (○) or stimulated (●) S-LB-I cells. The heavy line represents the standard for GM-CSF protein.

Table 1. Increased GM-CSF production by S-LB-I cells stimulated with PHA plus PMA

Cells	GM-CSF, ng/ml			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Nonstimulated	22.2	37.0	48.5	14.0
Stimulated	111.2	107.5	360.8	99.5

S-LB-I cells were stimulated by incubation with PHA (0.5% in Exps. 1–3; 2.0% in Exp. 4) plus PMA (10 ng/ml in all experiments). Medium was harvested at 20 hr from stimulated and control (nonstimulated) cells, and GM-CSF was determined by radioimmunoassay (see *Materials and Methods* and Fig. 2). The experiment was repeated four times. Determinations were done once for each experiment. Standard provided internal control.

stimulated −pCSFPI-transfected cells. The overall enhancement in five experiments was ≈5-fold, as determined by liquid scintillation counting, similar to the increases seen in GM-CSF protein (see Table 1).

Previous studies on interleukin 2 (IL-2) expression showed that treatment of T cells with the OKT3 monoclonal antibody and PMA mimics T-cell activation, whereas no IL-2 production was observed when Jurkat cells were cultured with either OKT3 or PMA alone over a wide range of doses (26). Based on this observation, cells were stimulated with 1:100 and 1:400 dilutions of OKT3 plus 50 ng of PMA per ml. Increased expression of +pCSFPI and not −pCSFPI was observed (Fig. 4). pSVcat (kindly provided by J. Banerji), containing the SV40 enhancer and promoter, was used as a positive control for transfection efficiency; no increased expression of pSVcat was seen when S-LB-I cells were stimulated with PHA plus PMA or with PMA alone (data not shown). BUG-CAT, containing the SV40 promoter and a nonfunctional immunoglobulin sequence, was used as a negative control; no CAT activity above basal level was detected in PHA/PMA-stimulated or nonstimulated S-LB-I cells (data not shown).

Cell-Type Specificity of GM-CSF Upstream Regulatory Sequences. To determine whether the 660-bp fragment is functional in non-T cells, +pCSFPI was transfected into PR, a human B-lymphoblastoid line (20). No CAT activity was seen when transfected cells were stimulated with lipopolysaccharide, pokeweed mitogen, or PMA, using pSV2cat as a positive control for transfection (data not shown).

DISCUSSION

We report here the identification of a region in the GM-CSF gene that acts in cis to regulate its expression in activated T cells. Thus far, GM-CSF appears to be a cell-specific factor. The HTLV-transformed cell lines HuT 102 (27), J-LB-I, S-LB-I, Mo, and C10-MJ2 (7) all showed basal production of GM-CSF RNA. Stimulation by PHA/PMA increases the level by as much as 10-fold (ref. 7 and Fig. 1). The increase in GM-CSF mRNA is accompanied by increased GM-CSF protein in conditioned medium, as detected by radioimmunoassay (Fig. 2).

Although peripheral blood lymphocytes showed no constitutive GM-CSF mRNA and a marked increase upon PHA/PMA stimulation (7), none of the currently available techniques efficiently transfect cloned DNA into primary T cells. We therefore chose to study GM-CSF induction in HTLV-transformed T-cell lines. The constructs we prepared contained 660 nucleotides 5′ from the first ATG of the GM-CSF gene, linked in both orientations relative to the bacterial CAT gene. The production of CAT in the constructs was shown to depend on the GM-CSF promoter and regulatory sequences.

Our findings suggest that the 660-bp fragment contains cis-acting regulatory functions. Only stimulated cells

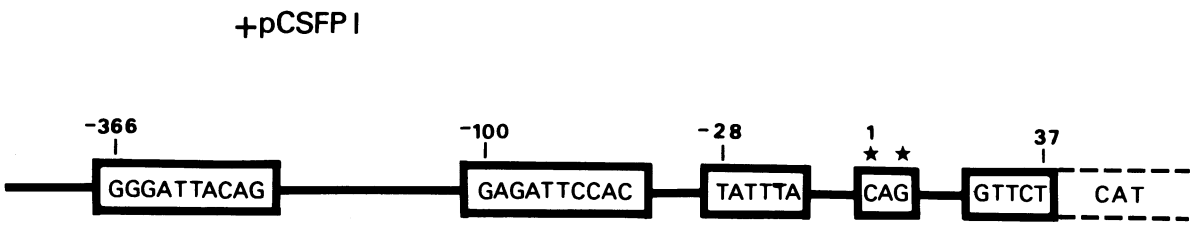

FIG. 3. Schematic diagram of the +pCSFPI plasmid. The heavy line represents the 660-bp fragment of GM-CSF, and the dotted box represents the CAT gene. Boxes at −336 and −100 represent the lymphokine decanucleotide consensus sequence found in the GM-CSF gene. The "TATA box" (at −28) and transcriptional initiation sites (stars) are also indicated.

transfected with the 660-bp fragment in the positive orientation (+pCSFPI) showed CAT activity above baseline in the assays. Nonstimulated and stimulated −pCSFPI-transfected cells, with the 660-bp fragment in the negative orientation, showed no CAT activity. Enhanced CAT activity in +pCSFPI-transfected cells after stimulation is not due to an increase in nonspecific cellular transcriptional and translational factors. pSVcat, containing the non-cell-specific SV40 enhancer and promoter, transfected into S-LB-I showed no detectable difference in CAT activity of nonstimulated vs. stimulated cells. Interestingly, PMA alone was able to somewhat enhance the expression of GM-CSF in S-LB-I cells. Peripheral blood lymphocytes and Jurkat cells have previously been shown to require two signals for activation (26). It is possible that HTLV infection enables the cells to bypass the requirement for the stimulus provided by PHA. Although HTLV-infected cells constitutively produce GM-CSF, no difference was detected in the background level of CAT activity between nonstimulated +pCSFPI- and −pCSFPI-transfected S-LB-I cells. Thus, the constitutive production of GM-CSF in HTLV-infected cells may be mediated via sequences that are not in the 660-bp fragment. Our results also suggest that the GM-CSF regulatory sequence is cell-specific, since no CAT activity was detected in pokeweed mitogen-, endotoxin (lipopolysaccharide)-, or PMA-stimulated +pCSFPI-transfected Epstein–Barr virus-transformed B cells. Recent work has shown that endothelial cells and some types of fibroblasts can be induced by IL-1 or tumor necrosis factor to produce GM-CSF (28, 29). It will be of interest to determine whether the sequences contained within the 660-bp fragment are involved in the expression of GM-CSF in these cell types.

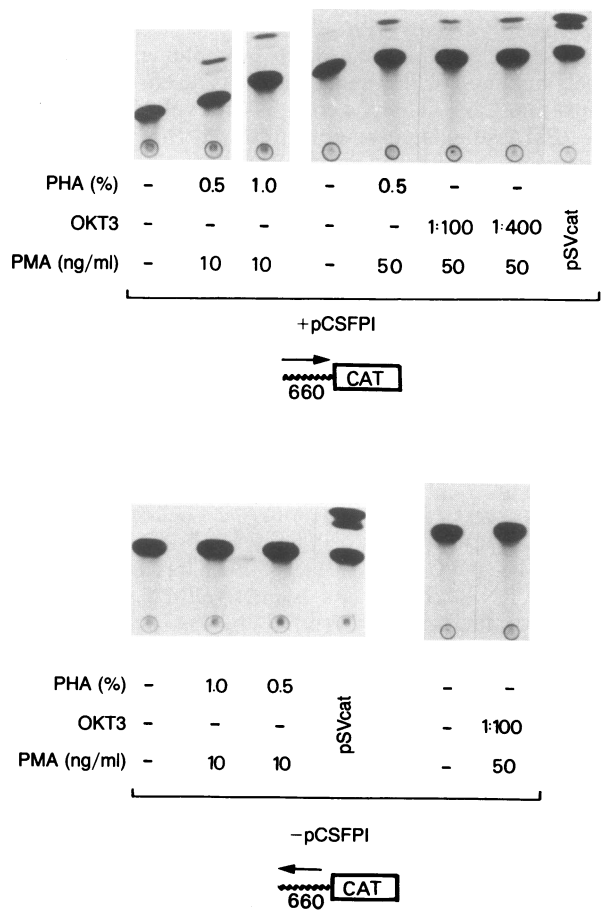

FIG. 4. Expression of CAT enzyme activity in S-LB-I cells. Migration of [^{14}C]chloramphenicol is from bottom to top. The acetylated forms of chloramphenicol are represented by the upper bands. In five experiments, the average conversion by stimulated relative to nonstimulated +pCSFPI-transfected cells was ≈5-fold.

We have also determined the transcription initiation ("cap") site of GM-CSF. The data indicate that transcription initiates at two different sites, nucleotides 28 (C) and 30 (G) from the putative "TATA box." This finding differs from that reported previously (30) for the human gene, where transcription initiates at nucleotides 23, 24, and 25 downstream from the TATA box. However, our finding correlates well with data obtained for the mouse gene (31). Also, our S1 analysis showed no evidence for another GM-CSF mRNA, as reported by Gough and coworkers (31, 32), that encodes a putative membrane-bound form of the protein.

It appears that GM-CSF expression is tightly regulated. It is possible to envision that T cells, when activated by physiological stimulators, produce cell-specific factors that trans-activate the GM-CSF gene via regulatory sequences situated within the 660-bp fragment. Comparison of the human and mouse GM-CSF showed sequences highly conserved in the region immediately upstream of the TATA box (30). It is interesting that the reported decanucleotide lymphokine consensus sequence (31) 5′ GRGRTTYCAY 3′ (R = A or G; Y = C or T), found in the murine IL-2, IL-3, and GM-CSF genes and human IL-2 gene, also occurs in two regions of the 660-bp human GM-CSF fragment. The consensus sequence may account for coordinated expression of these genes in activated T cells. The sequence situated at nucleotides −336 to −357 shows 80% homology, whereas the sequence at −100 to −91 shows 100% homology to the putative regulatory sequence (Fig. 3). It is possible that cell-specific factors produced during activation may act on these sequences to turn on GM-CSF production. Determination of the exact sequences with which T-cell-specific factors may interact will require the analysis of deletion and site-specific mutants of +pCSFPI.

We thank Shirley Quan and Sue Kaufman for technical assistance and Irvin Chen, William Wachsman, and Richard Gaynor for helpful comments on the manuscript. We thank Wendy Aft for excellent preparation of the manuscript. This work was supported by Public Health Service Grants CA40163, CA32737, and CA30388, and by American Cancer Society Grant JFRA-134 (J.C.G.).

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