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The structure and expression of the murine gene encoding granulocyte-macrophage colony stimulating factor: evidence for utilisation of alternative promoters

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Two overlapping genomic clones containing the murine granulocyte-macrophage colony stimulating factor (GM-CSF) gene have been isolated. On the basis of transfection experiments, we have established that a 9-kb *Bam*HI fragment from one of these recombinants encodes biologically active GM-CSF. As deduced from nucleotide sequence analysis, the GM-CSF gene comprises four exons encompassing 2.5 kb of genomic DNA. Primer extension analysis of GM-CSF mRNA identifies a transcriptional initiation site 35 bp upstream of a single translational initiation codon in-frame with the GM-CSF coding sequences and 28 bp downstream of a TATA promoter consensus sequence. Pre-GM-CSF molecules encoded by mRNAs originating from this promoter would include a hydrophobic leader sequence typical for a secreted protein. Intriguingly, sequences present at the 5′ end of a GM-CSF cDNA clone previously isolated in our laboratory are not contained within either of the genomic clones and must therefore be transcribed from a promoter located at least 10 kb 5′ of the main body of the gene. mRNAs transcribed from this alternative upstream promoter possess an additional initiating codon and potentially encode a pre-GM-CSF polypeptide with an atypical NH_2-terminal leader peptide. Comparison of the nucleotide sequence of the GM-CSF gene with that of other haemopoietic growth factor genes has revealed a common decanucleotide (5′-GPuGPuTTPyCAPy-3′) within their respective 5′-flanking regions which may be involved in their co-ordinate regulation.

Key words: haemopoietic growth factor/nucleotide sequence/multiple transcripts/inducer-responsive gene

Introduction

Haemopoiesis is the process whereby mature functional blood cells are derived from multipotential stem cells. The proliferation, differentiation and functional activation of haemopoietic cells *in vitro* is controlled by a group of glycoprotein regulators, the colony stimulating factors (CSFs) (Metcalf, 1984). Four murine CSFs have been identified that control the production of neutrophilic granulocytes and macrophages. Multi-CSF (Burgess *et al.*, 1980) [also known as interleukin 3 (Ihle *et al.*, 1982) and haemopoietic cell growth factor (Bazill *et al.*, 1983)] stimulates the progenitors of most haemopoietic cell lineages, while G-CSF (Nicola *et al.*, 1983) and M-CSF (Stanley and Heard, 1977) specifically stimulate committed progenitor cells of the granulocyte and macrophage lineages, respectively. However, GM-CSF (Burgess *et al.*, 1977) has an intermediate spectrum of activities, stimulating granulocyte, macrophage and eosinophil progenitor cells.

Several groups have examined the production of CSFs by various populations of primary lymphoid cells (McNeill, 1973; Parker and Metcalf, 1974) and more recently the cloned lines of T cells (see Kelso and Metcalf, 1985, and references therein). While many of these cells express constitutively low levels of various CSFs, the synthesis of these regulators can often be induced by antigenic or lectin stimulation. Furthermore, the production of distinct regulators by individual cloned T-cell lines often shows similar production kinetics, raising the possibility that the genes encoding these CSFs are activated by a common sequence of events.

We have previously reported the isolation of partial GM-CSF cDNA clones from a library established using mRNA isolated from the lungs of endotoxin-treated mice (Gough *et al.*, 1984). More recently we have isolated (Gough *et al.*, 1985) a significantly larger GM-CSF cDNA clone, pGM3.2, from a library synthesized using mRNA from a cloned T lymphocyte line, LB3, in which the synthesis of GM-CSF (and Multi-CSF) is inducible with concanavalin A (Kelso *et al.*, 1984; Kelso and Metcalf, 1985). This cDNA contains the entire coding region for GM-CSF and, when incorporated into a eukaryotic expression vector and transfected into tissue culture cells, directs the expression of a factor with the full range of biological properties previously ascribed to highly purified preparations of GM-CSF (Gough *et al.*, 1985).

Using cloned cDNAs as hybridisation probes, we have shown that GM-CSF is encoded by a unique gene in the murine genome (Gough *et al.*, 1984). To examine the organisation of the GM-CSF gene, and as a prelude to studying the basis of its regulation, we have isolated the GM-CSF gene from a recombinant murine genomic library and determined its DNA sequence. Intriguingly, it appears that the GM-CSF gene includes two promoters giving rise to alternative mRNAs which encode pre-GM-CSF polypeptides with different N termini.

Results

Isolation and expression of the GM-CSF gene

A library of murine genomic DNA was screened using as a probe a fragment of DNA derived from a partial GM-CSF cDNA clone, pGM37 (Gough *et al.*, 1984). Two positively reacting clones, λGM-1 and λGM-4, were isolated. Analysis of these clones using various restriction endonucleases indicated that they overlapped extensively and together encompass ~20 kb of mouse chromosomal DNA (Figure 1).

To determine whether the genomic DNA contained within the recombinants included a complete GM-CSF transcriptional unit, we have made use of the expression system previously described by Mellon *et al.* (1981). The vector pSVOd includes an SV40 origin of replication and lacks the sequences known to inhibit replication of certain plasmid-based vectors in mammalian cells

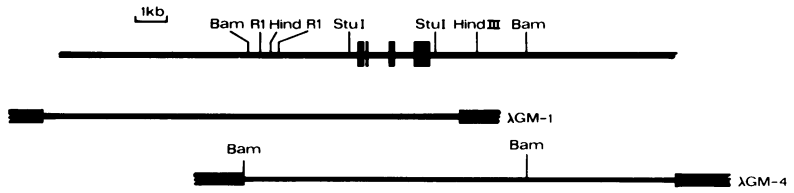

Fig. 1. Restriction enzyme cleavage map of the murine GM-CSF gene. Heavy boxes indicate the position of GM-CSF exons. The regions of chromosomal DNA contained within λGM-1 and λGM-4 are indicated.

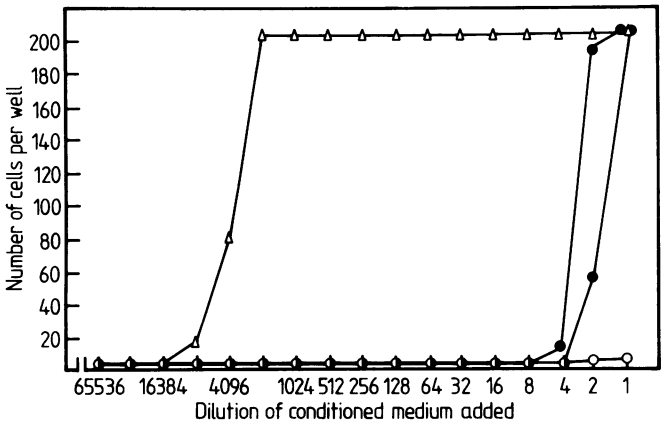

Fig. 2. Proliferation in microwell cultures of FDC-P1 cells stimulated by conditioned media (72 h) from COS cells transfected with pSVGM-5 (●) or pSVOd vector alone (○). Mouse lung conditioned medium as a standard source of GM-CSF was included as a control (△). Each point represents mean cell counts in duplicate wells scored after 2 days of incubation.

(Lusky and Botchan, 1981). Fragments of DNA may be inserted into pSVOd and, after transfection into Simian *COS* cells, which constitutively synthesise SV40 T antigen (Gluzman, 1981), such recombinants replicate to high copy number (Mellon *et al.*, 1981) thus providing for the expression of correspondingly high levels of gene product. In this system expression of the amplified gene requires an intact, autonomous transcriptional unit.

The 9 kb *Bam*HI fragment from λGM-4 (see Figure 1) was inserted into the corresponding site of pSVOd to generate pSVGM-5 and this construct transfected into *COS* cells. The culture medium harvested at 72 h included an activity capable of supporting proliferation of the CSF-dependent FDC-P1 cell line (Dexter *et al.*, 1980; Metcalf, 1985) (Figure 2). Furthermore, this activity was able to stimulate foetal liver progenitor cells to form colonies of maturing granulocyes and macrophages (data not shown). From these observations we concluded that the entire murine GM-CSF gene is contained within a 9-kb *Bam*HI genomic fragment and encodes biologically active GM-CSF.

Structure of the GM-CSF gene

Preliminary analysis of λGM-1 and λGM-4 revealed that those sequences which hybridised to various GM-CSF cDNA clones (Gough *et al.*, 1984, 1985) were located within a 2.7-kb *Stu*I fragment; the entire nucleotide sequence of this fragment was determined (Figure 3). By reference to the GM-CSF cDNA sequences (Gough *et al.*, 1984, 1985) we deduced that the GM-CSF gene consists of four exons which span ~2.5 kb of genomic DNA. A potential translation initiation codon occurs at position 262 within the first exon of the gene sequence and corresponds to an AUG identified within the GM-CSF cDNA clone pGM3.2 (Gough *et al.*, 1985).

The fourth exon encodes the C terminus of the GM-CSF protein preceding a translation termination codon at position 2282. A consensus polyadenylation signal occurs at position 2583, 18 nucleotides upstream of the polyadenylation site identified in two independently isolated cDNA clones (Gough *et al.*, 1984, 1985).

Identification of the GM-CSF transcriptional initiation site

To identify the initiation site for GM-CSF transcription within the gene sequence we have carried out primer extension analysis of GM-CSF mRNA. An oligonucleotide complementary to 30 nucleotides of the GM-CSF mRNA (position 363 – 392 in the gene sequence, Figure 3) was hybridised to mRNA from LB3 cells stimulated with concanavalin A and the primer extended using reverse transcriptase. The reaction products were separated on a denaturing polyacrylamide gel and their lengths determined by reference to a previously determined DNA sequence.

The major primer extension product of 165 nucleotides (track 1 in Figure 4) identifies the A residue at position 227 within the genomic sequence as the point of transcriptional initiation in stimulated LB3 cells. The sequence TATATAA, which is located 28 nucleotides upstream of this position, presumably forms part of the promoter complex for this GM-CSF transcriptional unit. No such extension product is evident when using mRNA from unstimulated LB3 cells (track 2, Figure 4) indicating that this promoter is activated during concanavalin A stimulation.

Discussion

Comparison with other haemopoietic growth factor genes

We have examined the sequences and structures of several haemopoietic growth factor genes for common features which may be involved in their co-ordinated regulation in various lectin or antigen-stimulated T lymphocyte cell lines (Kelso and Metcalf, 1985). In this context, there are two regions within the 5′-flanking region of the Multi-CSF gene (Miyatake *et al.*, 1985) that are homologous to a decanucleotide (5′-GAGATTCCAC-3′) located 78 bp upstream of the GM-CSF TATA promoter consensus sequence (Table I). One segment showed 80% homology with the GM-CSF decanucleotide and is located in the same relative position with respect to the TATA box while a perfectly conserved decanucleotide is present some 200 bp further 5′ of this position. While other short stretches of homology exist between the GM- and Multi-CSF genes, it is interesting that sequences related to the GM-CSF decanucleotide are also found in the 5′-flanking regions of both the murine (Fuse *et al.*, 1984) and human interleukin-2 genes (Fujita *et al.*, 1983) (Table I), genes whose expression is also induced in lectin or antigen-stimulated T cells (Gillis *et al.*, 1978). Thus, a consensus sequence 5′-GPuGPuTTPyCAPy-3′ can be deduced, which may play a role in the co-ordinated activation of these genes. It is noteworthy that a sequence resembling the above consensus sequence is also present, albeit in the reverse orientation, 136 nucleotides 5′ of the TATA box of the human γ-interferon gene

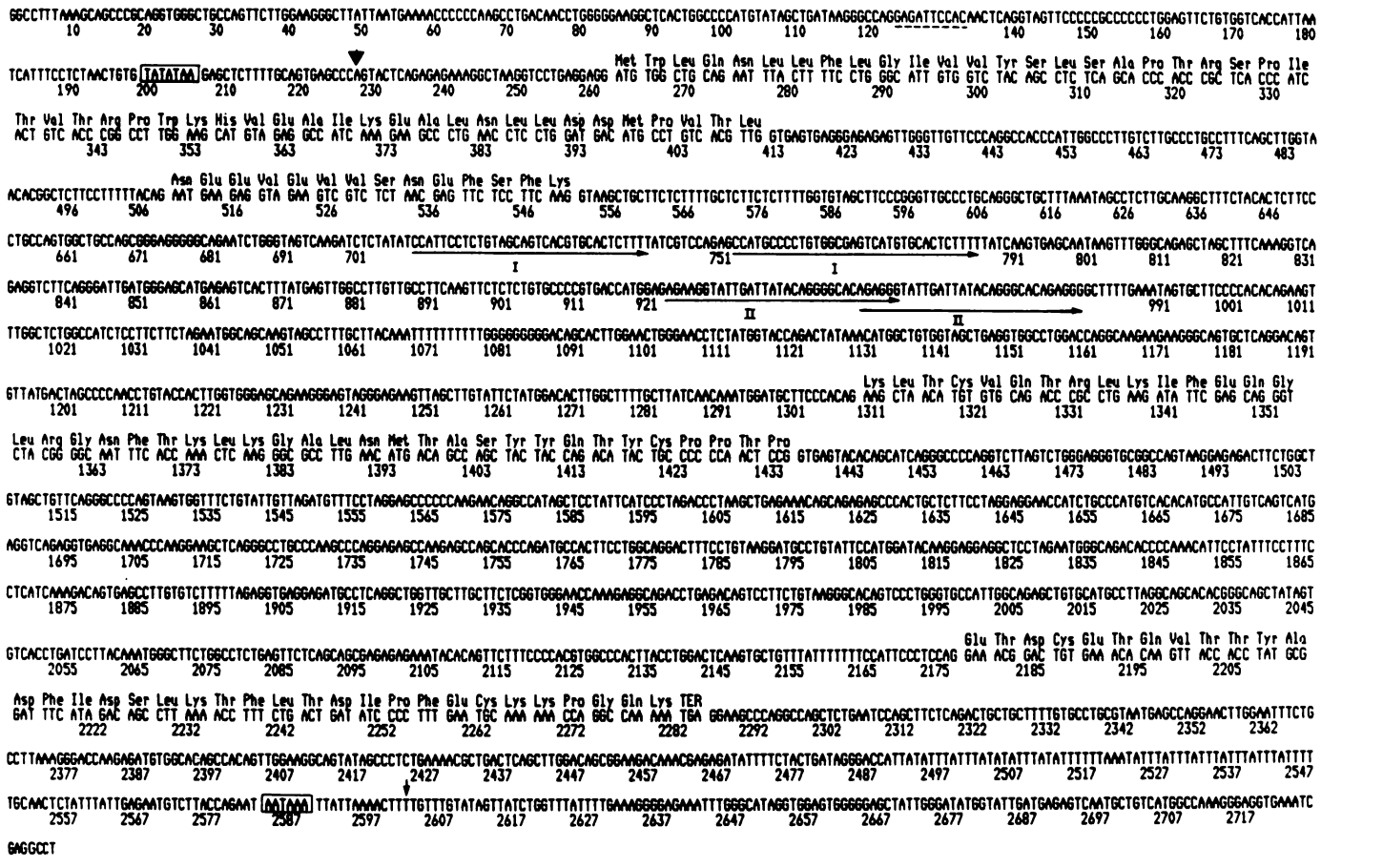

Fig. 3. DNA sequence of the 2.7-kb *Stu*I fragment encompassing the GM-CSF gene. The amino acid sequence encoded by the GM-CSF exons is shown above the DNA sequence. The TATATA promoter consensus sequence and polyadenylation consensus sequence AATAAA are boxed. The mRNA transcriptional initiation site is marked (▼). The point at the 3′ end where the cDNA and genomic sequence diverge is marked (↓). Two direct repeats (I and II), present in the second intron are underlined. The decanucleotide which is also present in the 5′-flanking region of the Multi-CSF (IL3) gene is indicated by a dotted line (see Table I).

(Gray and Goeddel, 1982) which is also subject to lectin-mediated activation (Yip *et al.*, 1981).

Our analysis has also revealed the presence of two independent direct repeats of ~33 bp and ~32 bp within the second intron of the GM-CSF gene. Interestingly, a 72 bp direct repeat has also been identified within the second intron of the multi-CSF gene (Miyatake *et al.*, 1985). However, the repeats within the GM-CSF gene show no significant primary sequence homology to those of the Multi-CSF gene. The importance of these repeats to the regulation of CSF gene expression remains to be established.

The GM-CSF gene encodes alternative mRNAs

Our transfection studies indicate that a 9 kb *Bam*HI fragment of mouse chromosomal DNA encodes biologically active GM-CSF. Indeed, all of the sequences within this fragment which hybridise to GM-CSF cDNA clones are contained within a 2.7-kb *Stu*I fragment. Sequence analysis of this fragment revealed that the GM-CSF gene comprises four exons spanning ~2.5 kb of DNA. Primer extension analysis indicates that the A residue at position 227 (Figure 3) corresponds to the major initiation site for GM-CSF mRNA synthesis in lectin-stimulated LB3 cells and is likely to be the promoter utilised in the autonomous expression of the 9 kb *Bam*HI fragment in our pSVOd/*COS* cell expression system.

Assignment of the GM-CSF transcriptional initiation site is, however, complicated by our analysis of the GM-CSF cDNA clone pGM3.2 (Gough *et al.*, 1985). While the DNA sequence of pGM3.2 is all but identical with the corresponding sequence of the GM-CSF gene, intriguingly, pGM3.2 has an additional segment of ~150 nucleotides at its 5′ terminus that is not encoded within the 2.7-kb genomic *Stu*I fragment and, indeed, not contained within λGM-1 or λGM-4 (data not shown). This sequence is likely, therefore, to be encoded at least 10 kb 5′ of the main body of the GM-CSF gene. The pGM3.2 cDNA and the genomic sequences diverge seven nucleotides downstream of the cap site at a position reminiscent of a splice acceptor site (Figure 5). Thus, pGM3.2 appears to correspond to an alternative transcript originating at an upstream promoter: sequences at the 5′ end of pGM3.2 presumably form part of an alternative exon(s) which is brought into juxtaposition with the GM-CSF coding sequences by a conventional splicing event.

There are several examples of eukaryotic genes that are associated with alternative promoters and subject to alternative modes of splicing. This arrangement provides a versatility for differential gene activation during differentiation (Young *et al.*, 1981) and for the generation of functionally distinct polypeptides from a unique gene. Mechanistically this can be achieved by alternative exon selection from a single precursor RNA (King and Piatigorsky, 1983; Nawa *et al.*, 1984; Rozek and Davidson, 1983; Early *et al.*, 1980; Maki *et al.*, 1981) or by the differential initiation of transcription followed by a different mode of splicing (Nabeshima *et al.*, 1984; Jonas *et al.*, 1985).

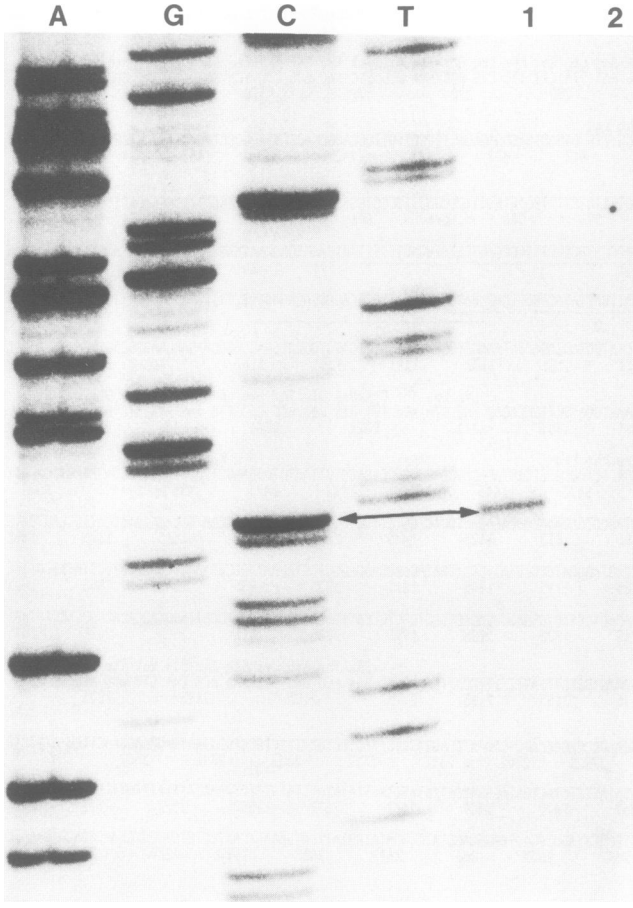

Fig. 4. Primer extension analysis on LB3 mRNA. A 5′ ^{32}P-labeled oligonucleotide complementary to part of the first exon (position 363–392, see Figure 3) was hybridised to cytoplasmic mRNA from LB3 cells and extended with reverse transcriptase as described in Materials and methods. The products were fractionated on a 7 M urea:8% polyacrylamide gel. **Lane 1,** poly(A)-containing RNA from concanavalin A-stimulated LB3 cells; **lane 2,** poly(A)-containing RNA from unstimulated LB3 cells. **Lanes A, G, C** and **T** show part of the sequence of an unrelated fragment of DNA of known size.

Table I. Homologies in the 5′-flanking regions of various haemopoietic growth factor genes

Factor	Sequence	Location[a]
Murine GM-CSF	5′-G A G A T T C C A C-3′	−78
Murine Multi-CSF (i)	5′-G A G A T T C C A C-3′	−265
Murine Multi-CSF (ii)	5′-G A G G T T C C A T-3′	−84
Murine Interleukin-2	5′-G G G A T T T C A C-3′	−175-1
Human Interleukin-2	5′-G G G A T T T C A C-3′	−172
Consensus	5′-G Pu G Pu T T Py C A Py-3′	

[a]Location corresponds to the distance between the 5′ nucleotide of the region of homology and the 5′ nucleotide of the TATA promoter consensus sequence.

What is the significance of alternative GM-CSF transcripts? Since different promoters must be utilised in the generation of these mRNAs, and as each mRNA encodes a distinct polypeptide, it is of interest to consider the importance of the promoter *per se* or, alternatively, of the inclusion of a supplementary exon(s) encoded by variant mRNAs which occurs as a consequence of alternative promoter usage. Since many T cell lines are known to constitutively express low levels of various CSFs

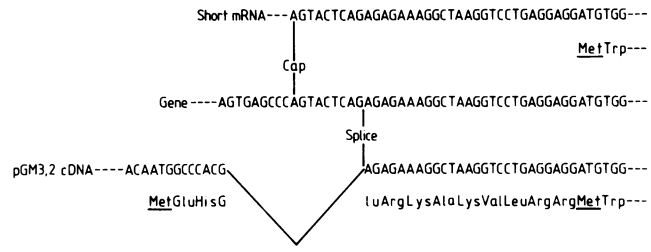

Fig. 5. DNA sequence of the GM-CSF gene (middle line) and alternative mRNAs. Upper line (short mRNA) shows the predicted nucleotide sequence of an mRNA initiated at the major cap site close to the main body of the gene (see Figure 3). The initiating methionine residue is shown below the sequence. DNA sequence of pGM3.2 cDNA (lower line) diverges at a potential splice junction. The supplementary 5′ exon(s) is encoded at least 10 kb upstream of this position. The deduced amino acid sequence is shown from the 5′-proximal AUG located in the supplementary exon. Thus, a protein initiated at the upstream AUG would have an extra 12 amino acids at the N terminus of its leader sequence.

(Kelso *et al.*, in preparation) and since their synthesis can be induced following exposure of the cells to lectin or antigen (Kelso *et al.*, in preparation; Kelso and Metcalf, 1985), it is possible that one of the promoters associated with the GM-CSF gene controls constitutive expression while the other might be inducible through its interaction with regulatory signals originating from the cell surface. Whilst the effect of lectin stimulation on the activity of the promoter giving rise to the 'long' mRNA (corresponding to cDNA clone pGM3.2) remains to be determined ('long' mRNAs could not be unambiguously identified in our primer extension experiment), the present study clearly implicates the promoter adjacent to the main body of the gene, which gives rise to the 'short' mRNA, in GM-CSF induction in concanavalin A-stimulated LB3 cells.

However, alternative promoter usage may be fortuitous other than in providing a mechanism for the generation of different mRNAs and the distinct polypeptides they encode. Pre-GM-CSF molecules encoded by the 'short' mRNA include a single AUG preceding a hydrophobic leader sequence which would subsequently be cleaved to liberate mature secreted GM-CSF (upper line, Figure 5). By contrast, the 'long' GM-CSF mRNA (lower line, Figure 5) includes two potential initiating codons (Gough *et al.*, 1985). Since it is likely that the 5′-proximal AUG would initiate translation in accordance with the 'scanning model' proposed by Kozak (1978, 1984) (and indeed, this is supported by recent *in vitro* experiments, B.Dobberstein, unpublished observations), pre-GM-CSF molecules encoded by this mRNA would include an extended and atypical leader, with seven of the first 12 residues being charged (Gough *et al.*, 1985) (see Figure 5). We have previously speculated that the presence of the atypical NH_2-terminal leader peptide may result in the synthesis of a membrane-bound form of GM-CSF. Since the 'long' mRNA must be transcribed from an independent promoter, it is conceivable that the cell could regulate the type of GM-CSF synthesised (membrane-bound or secreted) at the level of promoter usage.

Interestingly, we have recently shown that the Multi-CSF gene also encodes two mRNAs whose structure and mode of splicing are strikingly similar to those derived from the GM-CSF gene (Gough *et al.*, in preparation). As in the case of GM-CSF, the Multi-CSF gene specifies a 'short' mRNA which initiates close to the main body of the gene and a 'long' mRNA which differs in the presence of a supplementary 5′ exon encoded at least 14 kb 5′ of the gene. The 'short' mRNA has a single initiation codon

and encodes a pre-protein with a typical hydrophobic leader sequence, whereas the 'long' mRNA includes two additional 'in-frame' initiation codons and specifies a pre-Multi-CSF molecule with an extended and atypical N-terminal leader peptide.

Whether the utilisation of alternative promoters by the GM-CSF and Multi-CSF genes is a mechanism for generating the different forms of CSF (membrane-bound or secreted), or whether it simply constitutes a mode of quantitative regulation for CSF production, remains to be determined. In either case the use of different promoters which leads to the synthesis of alternative mRNAs and polypeptides is likely to reflect an important level of control in the biology of haemopoiesis.

Materials and methods

Isolation and sequencing of the murine GM-CSF gene

A murine genomic library provided by Joe Sambrook (Cold Spring Harbor) was screened with a probe derived from the GM-CSF cDNA clone pGM37 (Gough *et al.*, 1984). Two distinct recombinants, λGM-1 and λGM-4, possessing the GM-CSF gene were isolated. A 9-kb genomic *Bam*HI fragment from λGM-4 was subcloned into the vector pSVOd (Mellon *et al.*, 1981) to give pSVGM5. Restriction mapping of this recombinant localized GM-CSF hybridising sequences to a 2.7-kb *Stu*I fragment. Restriction fragments encompassing this region were subcloned into M13 vectors (Messing and Vieira, 1982) and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). Approximately 70% of the sequence was determined from both strands and most of the remaining sequence determined at least twice using independent clones.

DNA transfection and biological assays

DNA transfections were performed essentially as described by Danna and Sompayrac (1982) with modifications as detailed elsewhere (Gough *et al.*, 1985; Dunn *et al.*, 1985). *COS* cell-conditioned media were assayed for CSF activity 72 h post-transfection using the factor-dependent cell line FDC-P1 originating from Mike Dexter, Manchester (Dexter *et al.*, 1980). The present subline proliferates in response to either GM-CSF or Multi-CSF (Metcalf, 1985). FDC-P1 assays were performed as previously described (Gough *et al.*, 1985).

Primer extension synthesis

A 30-base oligonucleotide (positions 363 – 392 in Figure 3) was labeled at the 5′ terminus using polynucleotide kinase and [γ-^{32}P]ATP. Hybridisation of 50 ng of this primer with 1 μg poly(A)-containing RNA was carried out at 65°C in 0.3 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA. Hybrids were ethanol precipitated and primer extension with reverse transcriptase carried out at 40°C for 1 h in 50 mM Tris-HCl pH 8.3, 20 mM KCl, 10 mM $MgCl_2$, 10 mM DTT and 1 mM of all four dNTPs. After ethanol precipitation, the products were denatured and electrophoresed on an 8% polyacrylamide gel containing 7 M urea.

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