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The Human Hematopoietic Colony-Stimulating Factors

STEVEN C. CLARK AND ROBERT KAMEN

The complementary DNAs and genes encoding the four major human myeloid growth factors—granulocyte colony-stimulating factor, macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and interleukin-3—have all been molecularly cloned. These DNA clones have proved valuable for studying the molecular biology of these important regulatory molecules as well as for the large-scale production of the recombinant growth factor proteins. These advances have led to a much better understanding of the role of the myeloid growth factors in regulating hematopoiesis in vivo that should soon find practical application in clinical medicine.

THE REGULATED PRODUCTION OF BLOOD CELLS IS ONE OF the most complex examples of multilineage differentiation and one of those most amenable to scientific investigation. A common set of pluripotent stem cells, residing mostly in the bone marrow, gives rise to the large numbers of red cells, neutrophils, basophils, eosinophils, monocytes, platelets, and lymphocytes circulating in the blood. As many of these blood elements are short-lived, they must be continually replenished. Moreover, the levels of mature cells can respond dramatically to environmental stress. For example, granulocyte counts can vary from approximately 5,000 per microliter in normal humans to more than 50,000 in cases of severe infection; this variation, which corresponds to the production of approximately 2×10^{11} cells, can occur within a matter of only a few days (*1*).

A key advantage in studying the hematopoietic differentiation system has been that the blood is a liquid tissue composed of unicellular components. The development, more than 20 years ago, of cell culture systems for the clonal growth of hematopoietic progenitor cells in semisolid media was the key to the establishment of the hematopoietic lineages and the discovery that cell division and differentiation are dependent on the continuous supply of highly specific protein factors which act as regulators of hematopoiesis (*2*) (Fig. 1). Because these proteins were initially identified through cell culture colony formation assays, they were named the hematopoietic colony-stimulating factors, or CSFs.

CSF research has recently been intensified by cellular and molecular biologists for reasons beyond a purely scientific fascination with the hematopoietic system. Disease states caused by hematopoietic dysfunction or hyperplasia (leukemias) are significant medical problems. Furthermore, the possibility that CSFs could function as regulators of blood cell production in vivo suggested broad therapeutic utility and thus attracted the attention of the biotechnology industry. As a result of highly synergistic interactions among academic investigators, clinical researchers, and the scientists and technologists within industry, the field has advanced remarkably since the last review of the subject in *Science* by D. Metcalf (*3*). Most significant to those of us whose involvement stems from biotechnology, CSFs have now progressed from the laboratory to the clinic.

Several different colony-stimulating factors were distinguished through careful analysis of the cell types found in hematopoietic colonies grown with various sources of growth factor activity (*2*). In the murine system, four major types were identified. Two of these proved to be relatively lineage specific; that is, colonies grown in the presence of granulocyte-CSF (G-CSF) were found to consist largely of neutrophilic granulocytes and their precursor cells (*4*), whereas those grown in the presence of macrophage-CSF (M-CSF, also known as CSF-1) consisted largely of macrophages (phagocytic cells derived from circulating monocytes) (*5*). In contrast, the colonies grown in the presence of multi-CSF (also known as interleukin-3 or IL-3) were occasionally found to contain many different cell lineages (*6*), whereas those found in cultures grown in the presence of granulocyte-macrophage-CSF (GM-CSF) were found to contain neutrophilic granulocytes, macrophages, eosinophils, and other cell types (*7*). These results are believed to define a hierarchy of progenitor cells along the various cell lineages as summarized in Fig. 1. In this model, G-CSF and M-CSF are postulated to support the growth and proliferation of only relatively late progenitors already

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committed to their respective lineages. GM-CSF, in contrast, is presumed to interact additionally with somewhat earlier progenitor cells that are still capable of differentiating into neutrophils, eosinophils, or monocytes. The multiplicity of activities attributable to IL-3 is believed to be a consequence of its ability to support the growth of cells from relatively early pluripotent progenitors to mature cells of multiple lineages (*6*). In the human system, a set of four analogous factors has been described, each having properties similar to those of the corresponding murine G-, M-, GM- or multi-CSF (*2, 3, 8*). It should be emphasized, however, that these are not the only hematopoietic growth factors. For example, erythropoietin is a regulatory growth factor for terminal erythrocyte development and its importance in regulating red cell production in vivo is already clearly established (*9*). Interleukin-2 (IL-2) is a growth factor for mature T and B lymphocytes (*10*) and is certainly important in regulating lymphocyte function in vivo. Several other regulatory molecules that have recently been described affect at least some hematopoietic cells in an as yet poorly understood manner. These include hematopoietin-1 (*11*), which is believed to induce stem cells to become responsive to other CSFs and has recently been shown to be one of the activities of interleukin-1 (IL-1) (*12*); interleukin-4 (IL-4, also known as B cell stimulatory factor-1 or BSF-1) (*13*) which is a growth factor for mast cells and probably acts in synergy with other factors in supporting the proliferation of myeloid progenitors and at least some T and B lymphocytes; and B cell stimulatory factor 2 (BSF-2) (*14*), which has recently been found to interact with a variety of cell types. Although all of these molecules are in themselves intriguing, for purposes of limiting the scope of this article, we will focus our attention on the recent advances in the molecular biology, functional analysis, and therapeutic application of the four major human colony-stimulating factors now known to regulate the production of cells of the myeloid lineages: G-CSF, M-CSF, GM-CSF, and IL-3 or multi-CSF.

Molecular Cloning of the Human CSF Complementary DNAs

The application of recombinant DNA methodology to produce large amounts of homogeneous colony-stimulating factors affords the most practical route to advance the study of their role in hematopoiesis and to assess their therapeutic potential. Several groups had set out to clone human, as well as mouse, CSF complementary DNAs (cDNAs). Each factor represented a major research project involving the cooperation of cell biologists, protein biochemists, and molecular biologists. In each case, the CSF protein was present in trace amounts in the natural source, thus rendering its biochemical isolation extremely difficult. The low level of protein also reflected in general a low level of CSF messenger RNA (mRNA) in the various tissue or cell line sources. Nevertheless, the molecular cloning of cDNAs for each factor has been achieved, and the corresponding genes have been identified and also cloned (see Table 1).

The cDNAs for G-CSF (*15*) and M-CSF (*16*) were identified through a structural approach: the natural hematopoietins were purified to homogeneity; the purified factors were submitted to structural analysis; and the resulting amino acid sequence information was used to predict the nucleotide sequence of several different regions of the respective mRNAs. Synthetic oligonucleotides based on these sequences were used as hybridization probes to identify the G-CSF cDNA and a portion of the M-CSF gene and subsequently a complete M-CSF cDNA. These sequences were shown to be correct by expression of the cDNAs in mammalian cells to yield their respective biological activities. For M-CSF, considerable manipulation of the initial cDNA clones was required to obtain the first functional example (*16*).

Table 1. The human hematopoietic colony-stimulating factors. The indicated size ranges for the different protein species are those observed when the factors are expressed in mammalian cells. The M-CSF proteins are homodimers of monomers of the indicated sizes. Both mRNAs can be detected in the different sources of this factor, but usually the 4.0-kb sequence predominates. The mRNAs for the other colony-stimulating factors have been identified in the indicated primary cells but in all cases only after activation with mitogens or cytokines such as IL-1.

Name	mRNA size (kb)	Protein size (kD)	Cellular sources	Hematopoietic lineages found in colonies
G-CSF	2.0	18–22	Monocytes Fibroblasts	n
GM-CSF	1.0	14–35	T cells Endothelial cells Fibroblasts	n,m,e,E,M
IL-3 (multi)	1.0	14–28	T cells	n,m,e,b,E,M
M-CSF	4.0 1.8	35–45 (×2) 18–26 (×2)	Monocytes Fibroblasts Endothelial cells	m

A more detailed examination of G-CSF cDNAs and the structure of the G-CSF gene has revealed that at least two pathways exist for splicing the primary transcript of this gene (*17*). The two mRNAs differ in that one contains an additional three codons resulting in a G-CSF protein that has three amino acids inserted between residues 35 and 36 of the shorter version of this protein. Sequence analysis of the G-CSF gene revealed that this insertion resulted from utilization of an alternative 5′ donor splice site in the squamous carcinoma cell line CHU-2. Preliminary experiments have indicated that the G-CSF protein having the additional three amino acids is substantially less active in supporting granulocyte colony formation than the shorter version of the molecule. It is certainly of interest to compare these molecules in a variety of bioassay systems to see if some of the different biological activities of G-CSF might arise from altered versions of the same gene product. However, it is also possible that the altered, less active form of the molecule is a consequence of aberrant splicing in the tumor cell line under study, and further work will be necessary to demonstrate the expression of the second G-CSF mRNA from primary cellular sources of this factor.

A similar but more complex situation occurred in the cloning of M-CSF cDNAs. The original cDNA clone was derived from a 1.8-kb mRNA expressed by the pancreatic carcinoma cell line Mia PaCa after phorbol ester induction, although M-CSF is constitutively expressed by these cells (*16*). Hybridization analysis of M-CSF mRNA from a variety of primary human cells and from other cellular sources of M-CSF subsequently demonstrated that a 4-kb message is the predominant transcript for this factor (*16, 18*). A comparison of the structures of the cDNAs corresponding to the 4- and 1.8-kb mRNAs revealed that the longer sequence contained an insertion of 894 nucleotides within the protein coding region as well as a substantially longer 3′ noncoding sequence. These differences result from alternative pathways of splicing of the primary M-CSF transcript (Fig. 2). Because the sequence of 894 residues contained within the protein coding region preserve the M-CSF reading frame, the two transcripts encode related but different polypeptides that are processed post-translationally to yield different forms of M-CSF, as discussed below.

The cDNAs for human GM-CSF (*19*) and IL-3 (*8*) were cloned by a different strategy. As has proved to be true for many growth factors and regulatory molecules, all of the CSFs are active at very low (picomolar) concentrations. This high intrinsic biological activi-

ty means that apparently "rich" natural sources of the CSFs in reality often contain only trace physical quantities of the factors, rendering their purification to homogeneity extremely difficult. However, this high intrinsic activity can be exploited by the molecular biologist to identify cDNA clones that direct the expression of the desired CSF when introduced into mammalian cells with appropriate expression vectors. The cDNAs for the human GM-CSF and the gibbon IL-3 were both identified in this manner. Both of these cDNAs were isolated by testing random pools of plasmid cDNAs for their ability to direct the expression of the desired biological activity when introduced into monkey COS-1 cells (*8, 19*).

The human analog of the murine multi-CSF or IL-3 proved to be the most elusive of the four human CSFs to identify. In retrospect, it is clear that this difficulty resulted both from a low level of nucleotide sequence homology with the murine IL-3 and from a low level of expression of the human factor by activated peripheral blood lymphocytes (PBLs). The low level of expression of IL-3 by stimulated human T cells makes it difficult to detect the biological activity of this factor in PBL-conditioned medium, which is a good source of human GM-CSF, a factor that shares many of the activities of IL-3 (*20*). In our previous work, we had developed a rapid proliferation assay for GM-CSF, in which the targets were primary leukemic cells isolated from patients with chronic myelogenous leukemia (CML). Using this assay, we discovered that the conditioned medium from a gibbon T cell line (UCD-144-MLA) contained high levels of a CML cell growth factor activity that was only partially neutralized by antibody against recombinant GM-CSF. Because the gibbon cell line was known to express high levels of GM-CSF, the DNA pools from the gibbon T cell cDNA library were screened for the ability to direct the expression by COS-1 cells of a CML growth factor activity that was resistant to the antiserum to GM-CSF. The cDNA identified in this screen proved to encode a multilineage CSF when tested with normal human bone marrow target cells, and this multilineage CSF was unrelated to GM-CSF. Computer analysis of the DNA sequence of the gibbon cDNA revealed that it shared low but significant homology with the sequence encoding murine IL-3 (49% at the nucleotide level; 29% at the amino acid level). The corresponding human gene was easily identified and cloned when the gibbon sequence was used as a hybridization probe. From the gene sequence, the human and gibbon proteins were found to differ in amino acid sequence at 11 positions.

The human and murine IL-3 genes have very similar structures (*8, 21, 22*). Both genes consist of five relatively small exons separated by

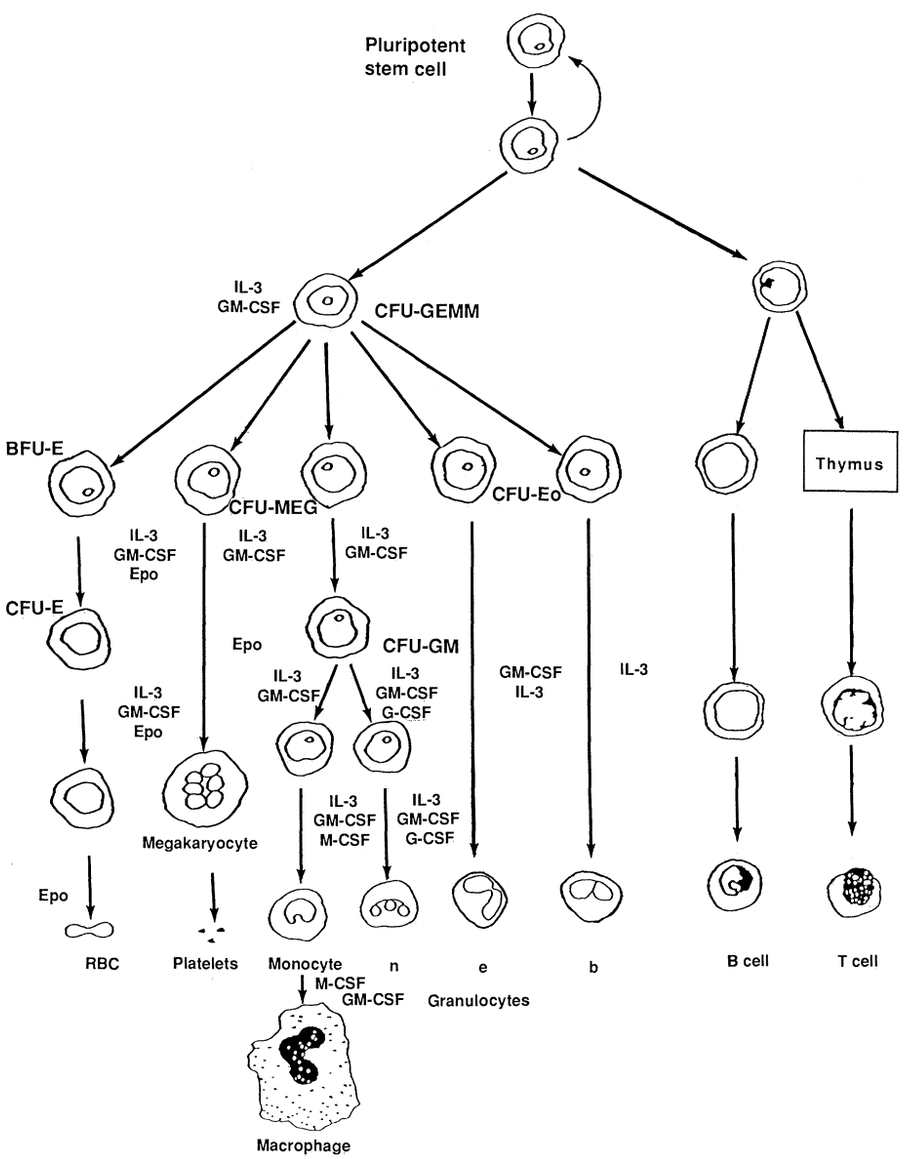

Fig. 1. Interactions of the CSFs with hematopoietic cells. The pathways of hematopoiesis are as described by Metcalf and Nicola (*4*). The different progenitor cells which are identified in the in vitro culture systems are CFU-GEMM (colony-forming unit, granulocyte-erythrocyte-monocyte-megakaryocyte), CFU-Meg (CFU-megakaryocyte), CFU-Eo (CFU-eosinophil), CFU-GM (CFU-granulocyte/monocyte), CFU-E (CFU-erythroid), and BFU-E (burst-forming unit–erythroid). The abbreviations for the hematopoietic lineages (as proposed at a UCLA symposium) are n, neutrophil; e, eosinophil; b, basophil; m, monocyte/macrophage; E, erythrocyte; and M, megakaryocyte. The interactions of the different CSFs with the various lineages are as indicated. These interactions are based on analysis of mature cells found in colonies grown in the presence of the CSFs as discussed in the text. The sites of action are intended to indicate that at least some but not necessarily all of the progenitors of that lineage are responsive to the indicated CSF. Erythropoietin (Epo) is essential for development of erythroid cells (*9*) and can promote the differentiation of megakaryocyte progenitors in vitro (*67*).

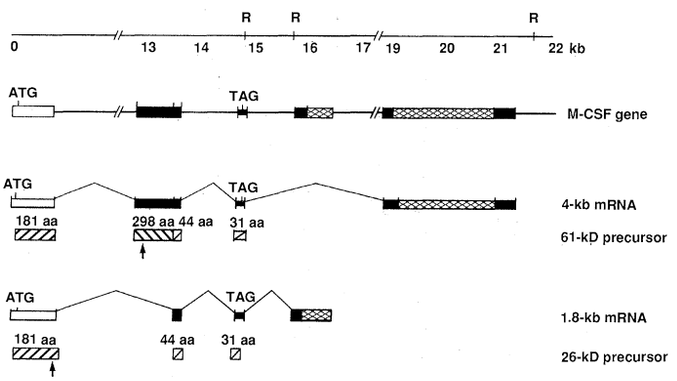

Fig. 2. Alternate pathways of expression of the M-CSF gene. The two different M-CSF mRNAs (1.8- and 4.0-kb mRNAs) arise from alternate splicing pathways as illustrated. The relevant structural features of the gene are illustrated at the top. The M-CSF gene consists of many exons which are distributed over 22 kb of DNA. The first 750 bases of either mRNA result from identical splicing together of many small exon sequences (not shown) that are located in the first half of the gene (12.5 kb). The differences in the structures of the two mRNAs result from alternative splicing of the four remaining exons [the heavy solid lines indicate exons whose complete sequence has been determined (*68*), and the hatched areas indicate regions that have not been completely sequenced and therefore could contain additional small introns]. The positions of the initiation (ATG) and termination (TAG) codons utilized by either transcript are as indicated. The major difference in structure results from a splicing event in the 1.8-kb mRNA in which the ultimate 5′ donor site from the first half of the gene is joined to a 3′ acceptor contained within and near the 3′ end (position 13.5 kb in the M-CSF physical map) of a large exon found in the 4-kb mRNA. This results in a deletion of 298 residues encoded by this exon from the coding region of the smaller transcript. The two transcripts also utilize different exon sequences to complete their respective 3′ noncoding regions as shown. The primary translation products of the two mRNAs have identical amino and carboxyl terminal domains (indicated by ▨) but differ by the 298-residue domain (indicated by ▩) as shown. The arrows indicate the locations where carboxyl terminal processing ultimately occurs to yield the related but distinct M-CSF polypeptides encoded by either mRNA species.

one large and three small introns. The nucleotide sequence homology shared by the human and murine genes is spread relatively evenly throughout the 3000 base pairs spanning the genes. The regions of highest homology are found in the putative promoter region (59%); and in the 3′ noncoding region of the fifth exon (59%). Computer analysis of the GM-CSF genes of man and mouse have provided similar results; the most highly conserved sequences are in the promoter region and 3′ noncoding sequence of the cDNA (*23*). The high degree of conservation in evolution of these noncoding sequences targets them as potentially important in the regulation of the expression of these genes. In retrospect it is perhaps not surprising that codon degeneracy and conservative functional relationships among amino acids allows protein-coding sequence to diverge more rapidly than regulatory sequences which are most likely recognized by sets of DNA-binding proteins.

Although human GM-CSF and human IL-3 have some common biological activities, some common structural features, and are both expressed in activated T cells, computer analysis of the gene sequences has failed to reveal any significant sequence homology shared by the two genes. Sequence elements common to many eukaryotic promoters are found in the 5′ flanking sequences of the GM-CSF (*23*) and IL-3 (*22*) genes and both 3′ noncoding regions are very rich in A · T base pairs, but a detailed comparison of these sequences suggests that they do not have a recent common origin in evolution. It is interesting, however, that both the human GM-CSF and IL-3 genes have been mapped to the same band on the long arm of chromosome 5 (*24*). This is in a region which has also been shown to contain the genes for M-CSF (*24*) and its receptor, the proto-oncogene, c-*fms* (*24–26*), as well as the genes for several other growth factors and growth factor receptors. The significance of this clustering of genes on chromosome 5 is not clear, but it is particularly intriguing that the genes for IL-3 and GM-CSF are very close together (at least in terms of chromosomal distances). It will be interesting to determine whether this proximity in any way relates to the regulation of their expression. Deletions on the long arm of chromosome 5 [del(5)q] are frequently observed in patients with myelodysplastic syndrome and acute non-lymphocytic leukemia secondary to cytotoxic therapy and in refractory anemia characterized as "5q-syndrome" (*27*). The localization of the genes for myeloid growth factors and their receptors in this chromosomal region may imply a causal relation of a nature yet to be elucidated.

Regulation of CSF Gene Expression

A key feature of the model for growth factor regulation of hematopoiesis is that the functional hematopoietins must be produced and released by appropriate cells, so that they reach their target progenitor cells in timely fashion. The availability of the DNA probes for the four CSFs has made it possible to begin to study the expression of the different CSF genes in different cell types at the mRNA level. As mentioned earlier, steady-state blood cell production is normally maintained in the body at a continuously high rate. However, the levels of blood cells in circulation can change rapidly in response to specific signals such as infections. Thus far, the pattern of expression of the CSF genes in different cell types is consistent with the proposal that the different cloned hematopoietins are involved in emergency responses, but the evidence for their importance in maintaining steady-state hematopoiesis is incomplete. These conclusions are drawn from the observations that normal cells that constitutively express detectable levels of mRNA for G-CSF, GM-CSF, or IL-3 have not been identified (only a small number of cells have been examined for IL-3 expression). However, several different cell types have been found to express different CSF genes following activation. Monocytes, for example, when stimulated with endotoxin, interferon-γ, or phorbol esters have been shown to contain high levels of mRNA for G-CSF and M-CSF but not for GM-CSF (*28*). Normal activated T cells, in contrast, express relatively high levels of GM-CSF mRNA (*18*) and probably low levels of IL-3 mRNA (*8*) but do not express the mRNAs for either G- or M-CSF (*28*). Several investigators have recently found that IL-1 and tumor necrosis factor (TNF) activate the expression of G-CSF and GM-CSF in various cell types, including endothelial cells and fibroblasts (*29*). These cell types are present in bone marrow stroma, and, indeed, primary human stromal cells can be induced to produce G- and GM-CSF mRNA (*30, 31*). These observations support the idea that infections which result in T cell or monocyte activation will lead directly to the production of GM-CSF and IL-3 by the activated T cell, to the production of G-CSF and M-CSF by the activated monocyte, and indirectly to the production of GM-CSF and G-CSF by a variety of cells in response to different lymphokines (interferon-γ) and monokines (IL-1 and TNF). However, the failure to detect the expression of these genes in normal unstimulated primary cells leaves open the question of the role of GM-CSF, IL-3, and G-CSF in maintaining steady-state hematopoiesis. It is possible that stromal cells in the microenvironment of the marrow produce low levels of these or other as yet ill-defined growth factors that maintain the proliferation of the progenitors; such production could be regulated by monokines like IL-1. Alternatively, cellular interactions between stromal and hematopoietic cells in the marrow may play the crucial regulatory role in maintaining hematopoiesis in

the marrow (*32*). Further work will be necessary to determine the interactions of cells and growth factors in the marrow.

In contrast to the other CSFs, the 4-kb mRNA for M-CSF is readily detectable in a variety of primary tissues and cells (*16, 18*). The seemingly promiscuous tissue expression of the M-CSF gene may reflect one of the roles of this molecule in vivo. If M-CSF is a survival factor for monocytes and macrophages, the constitutive production of this factor by a variety of cells in tissues may be necessary to support the relatively long-term survival of monocytes as they leave the circulation, migrate into tissues, and develop into macrophages.

Structure of the Recombinant CSFs

Considerable effort has been devoted to the purification of the natural human GM- (*33*), G- (*34*), and M-CSFs (*35*), while the human IL-3 protein has been detected only as an activity present in medium conditioned by lectin-stimulated peripheral blood lymphocytes (PBLs). These studies plus immunochemical analysis of natural GM-CSF (*36*) have confirmed that the recombinant factors produced in mammalian cells have structures very similar to the natural molecules. Because of their availability, the most detailed structural studies have been performed with the recombinant CSFs. As mentioned previously, human IL-3 (*8*) and GM-CSFs (*33*) have several common structural features. Both factors are processed at the amino terminus to yield mature polypeptides beginning with the dipeptide Ala-Pro and having an expected mass of 14 to 15 kilodaltons. These molecules contain internal disulfide bridges (GM-CSF, two, and IL-3, one), which are important in the maintenance of the structure of the factors. Both factors are complex glycoproteins, each having two potential sites for asparagine-linked carbohydrate. Through pulse-labeling studies of mammalian cells expressing high levels of either GM-CSF or IL-3, we have found that both recombinant factors are extremely heterogeneous in size (Fig. 3), with molecular species visible in the 14- to 30-kilodalton size range. Because the expected size of either mature polypeptide is 14 to 15 kD, the carbohydrate content of the molecules ranges up to 50% of the mass of the protein. Studies with mutant GM-CSFs have demonstrated that this great size heterogeneity is attributable to the state of occupancy of the asparagine-linked carbohydrate sites: the largest species of GM-CSF have both sites occupied, intermediate species only a single site occupied, and the smallest forms have no *N*-linked oligosaccharide (*36*). The recombinant GM-CSF expressed in Chinese hamster ovary cells is also modified in several positions by the addition of *O*-linked oligosaccharide, a modification which contributes additional heterogeneity to the protein (*37*). Natural GM-CSF is probably also modified by *O*-glycosylation, but this has not been rigorously proven. The pattern of heterogeneity observed with human IL-3 derived from COS-1 cells (Fig. 3) is similar to that of GM-CSF, suggesting that analogous carbohydrate modifications account for the size distribution of human IL-3.

The function of the extensive carbohydrate modification of GM-CSF and IL-3 is unknown. In the case of GM-CSF, it is clear that the oligosaccharide moieties interfere with the growth factor activity of the polypeptide measured with in vitro assays. Indeed, the most heavily glycosylated GM-CSF is less than one-tenth as active as the smallest form of the molecule lacking asparagine-linked carbohydrate (*38*). Using metabolically labeled GM-CSF separated according to size, we found in rats that the different size classes of the protein are cleared from circulation with different kinetics (*36*). In this study, the rate of clearance of GM-CSF into the kidney was not affected by the occupancy of the *N*-linked carbohydrate sites (half-time, approximately 65 minutes). However, the rate and extent of the distribution of the GM-CSF throughout the fluids and tissues of the rat were substantially dependent on glycosylation. GM-CSF having both asparagines modified displayed single-phase kinetics (half-time, 65 minutes), which suggests that the rate of distribution of this form of the molecule is slow relative to its rate of clearance by the kidney. In contrast, molecules lacking *N*-linked sugar appeared to distribute quickly (70% to 80% lost from circulation, with an apparent half-life of 4 to 5 minutes) from the bloodstream. Intermediate species having a single *N*-linked site modified displayed an intermediate rate of distribution. Although the significance of these findings is not clear, it is interesting that the distribution of GM-CSF molecules in vivo can be influenced by the extent of glycosylation. In this regard, it should be mentioned that several other growth factors and regulatory molecules including interferon-γ, human IL-3, and BSF-2 also have two sites for addition of *N*-linked carbohydrate. Important exceptions are IL-2 and G-CSF, glycoproteins whose carbohydrate is only attached through *O*-linkages. The ability to produce the different forms of all of these factors now makes it possible to address these questions experimentally. The answer will bear on the relative merits of bacterial or mammalian expression for the production of recombinant CSFs intended for human use.

Because the primary sequence of human G-CSF does not include any sites for asparagine-linked glycosylation, the recombinant protein produced in mammalian cells is much less heterogeneous in size than either GM-CSF or IL-3 (Fig. 3). The observed size heterogeneity is due to *O*-glycosylation (*15*). The primary sequence of G-CSF contains four cysteine residues which are presumed to form two specific disulfide bridges. Large quantities of the mature human G-CSF lacking carbohydrate have been produced in *Escherichia coli* and this material has proved to be highly active both in vitro and in vivo (*39*).

The biosynthesis of human M-CSF is easily the most complex of all of the colony-stimulating factors. The mature protein is a disulfide-linked homodimer processed from a larger precursor (*16, 18*). Fortunately, mammalian cells including Chinese hamster ovary cells or COS-1 cells are fully capable of all of the required post-translational modifications. Because of this, it is possible to dissect

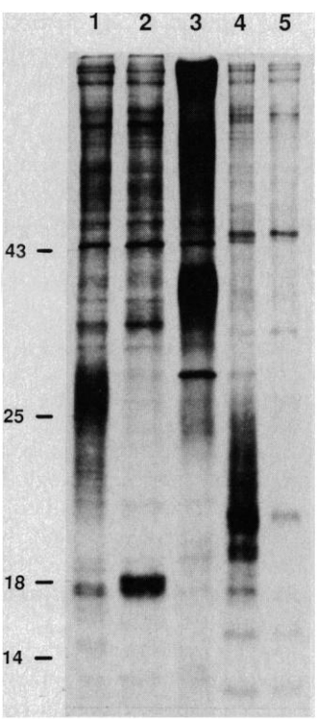

Fig. 3. Expression of the recombinant human CSFs in mammalian cells. SDS-polyacrylamide gel analysis of ^{35}S-labeled conditioned medium reveals the size distribution of the different CSFs expressed by mammalian cells. The size heterogeneity is a consequence of variation in both the extent of glycosylation of each molecule and in the structure of the added carbohydrate. Conditioned media from Chinese hamster ovary (CHO) cells engineered to express human GM-CSF (lane 1), human G-CSF (lane 2), and human M-CSF from the 4-kb mRNA (lane 3) were analyzed by SDS-polyacrylamide gel electrophoresis after reduction as described (*8*). Conditioned media from COS-1 cells transfected with a plasmid (Y3) designed for expression of human IL-3 (lane 4) or transfected with the expression vector pXM (lane 5) were analyzed in parallel on the same gel. The relative mobilities of reference proteins were as indicated.

the mechanism of M-CSF biosynthesis through analysis of the expression of M-CSF mutants as well as to produce substantial amounts of the protein for detailed structural analysis. The M-CSF system is further complicated by the observation that at least two differentially spliced mRNAs are expressed from a common gene and that each of these encode different M-CSF precursors. The larger precursor (61 kD) encoded by the principal 4.0-kilobase mRNA contains a sequence of 298 amino acids inserted within the coding sequence of the smaller (26-kD) precursor that is encoded by the 1.8-kb mRNA (Fig. 2) (*18*). This sequence is precisely inserted between residues 181 and 182 of the small precursor in such a way that the reading frame is maintained and both precursors share a common carboxyl terminal sequence of 75 amino acids. The two M-CSF precursors share a common amino terminal sequence that is processed by the removal of the first 32 residues. The 61-kD precursor is additionally processed at the carboxyl terminus by removal of approximately 294 residues to yield a mature polypeptide of 223 residues with an expected molecular weight of 26 kD. We speculate that the smaller precursor is processed at the carboxyl terminus by removal of about 80 residues leaving a mature subunit of about 145 residues and having an expected molecular weight of about 16 kD. Both forms of M-CSF are further modified by *N*-glycosylation and at some point they associate to form dimeric molecules that are the active forms of the hematopoietin. Thus at least two forms of M-CSF exist: a 70- to 90-kD glycoprotein comprising a dimer of a 35- to 45-kD subunit (about 223 amino acids) and a 40- to 50-kD glycoprotein comprising a dimer of a 20- to 25-kD subunit (about 145 amino acids).

Two different sizes of M-CSF have been purified from natural sources (*35, 40*). These sizes correlate well with those of the recombinant M-CSFs. In fact the amino acid sequence of the larger form of the natural hematopoietin (purified from human urine) corresponds quite precisely to the 70- to 90-kD recombinant factor encoded by the 4-kb mRNA (*18*). It is not yet clear whether the smaller form of M-CSF in natural sources is the proteolytic degradation product of the larger M-CSF molecule or a distinct protein secreted from specific cells; indeed, mild protease treatment of 70- to 90-kD M-CSF generates a molecule similar in size to the smaller form of the protein (*41*).

The sequence of 75 amino acids that is found at the carboxyl terminus of both M-CSF precursors contains a hydrophobic region comprising 23 residues followed by a sequence of charged residues (*16*). This structure is reminiscent of a transmembrane domain and an anchor (*42*), suggesting that the M-CSF precursors might be inserted into membranes. The mature forms would then be generated by proteolytic cleavage, either within the cell or after transfer of the precursor to the cell surface. Although M-CSF processing may normally follow this pathway, several mutant cDNAs that lack this transmembrane sequence have been found to express and secrete M-CSF efficiently when transfected into COS-1 cells (*43*).

Biological Activities of the CSFs

The molecular cloning and the subsequent production of all four recombinant CSFs has permitted detailed analysis of the biological activities of the respective factors. In general, the biological activities of the human recombinant G-, GM- and M-CSF were found to agree with those of the natural hematopoietins. The activities of the recombinant primate IL-3, a factor not previously identified from natural sources, are similar to those of the murine IL-3.

Recombinant human G-CSF is a lineage-specific hematopoietin that directly supports the proliferation of colonies consisting primarily of neutrophils (*15*). The colonies, in general, appear more quickly in culture than those supported by either GM-CSF or IL-3, so that peak numbers are attained by day 7 in culture, after which the number declines. Occasionally, other types of colonies are found in G-CSF–supported cultures, but these seem to arise from indirect effects mediated by accessory cells (*11*). Recombinant G-CSF also profoundly influences the biological activities of mature neutrophils, for example, by enhancing their ability to produce superoxide anions in response to the bacterial peptide f-Met-Leu-Phe, to kill tumor targets through antibody-dependent cellular cytotoxicity, and to enhance their ability to phagocytize particles (*44*). In addition, G-CSF appears to promote the differentiation of some but not all myeloid leukemic cells (*42*). These activities are consistent with the idea that G-CSF is a growth and differentiation factor for a population of late progenitor cells committed to the neutrophil lineage and is a priming factor for the resulting mature cells.

Recombinant human GM-CSF, like its natural counterpart, has a broad range of activities. In contrast to G-CSF, GM-CSF supports the proliferation of macrophage and eosinophil colonies as well as colonies containing both neutrophils and macrophages, although single-lineage colonies predominate (*45, 46*). These results confirmed that recombinant human GM-CSF displays the same activities as those ascribed to the natural CSF designated CSF-α (*45*). In addition, the cloned factor has some ability in the presence of erythropoietin to support the proliferation of erythroid and megakaryocytic progenitors (*20, 46*) as well as progenitors that give rise to mixed colonies having all myeloid elements, including erythroid cells. These results agree with the proposal that GM-CSF interacts with earlier progenitors than G-CSF does. GM-CSF is also a survival and activating factor for the end cells derived from GM-CSF–responsive progenitors (*47*). Thus, GM-CSF enhances the biological activities of neutrophils, eosinophils, and macrophages (*47, 48*), in contrast to G-CSF, which is selective for neutrophils (*44*). GM-CSF is also a growth factor for the leukemic cells from many myeloid leukemia patients, both chronic and acute (*49*). Although there is little evidence that the aberrant expression of the GM-CSF gene is involved in the generation of acute myelogenous leukemia, several patients have been identified whose blast cells are autocrine for GM-CSF, suggesting that within a population of leukemic cells there may be a selective advantage for cells expressing GM-CSF (*50*).

The ability of the recombinant gibbon IL-3 (the gibbon and human factors share 93% homology and should have identical biological activities) to support hematopoietic colony formation has proved to be similar to that of the murine factor. Recombinant gibbon IL-3 supports the proliferation of myeloid progenitors including those of erythrocytes, neutrophils, eosinophils, basophils, macrophages, and megakaryocytes (*46, 51*). Numerous colonies comprising multiple lineages have been identified in these cultures. The factor also supports the proliferation of greater numbers of erythroid colonies than does GM-CSF, further supporting the model that primitive multilineage progenitors are responsive to IL-3 but not to GM-CSF. Addition of GM-CSF to cultures grown in the presence of IL-3 did not result in enhanced colony formation, suggesting that most if not all GM-CSF progenitors also respond to IL-3 (*51*). In contrast, addition of G-CSF to the IL-3–containing cultures resulted in substantially greater numbers of neutrophil colonies than found with IL-3 alone, without depressing colony formation by other cell types. This result implies that there may be distinct subsets of neutrophil precursors that are responsive to either IL-3 or G-CSF. It is interesting in this regard that the recombinant IL-3 has thus far proved to be an activating factor for eosinophils but not neutrophils (*52*).

A further demonstration that the gibbon IL-3 supports the growth and differentiation of early pluripotent cells comes from

analysis of human 21-day blast-cell colonies from cultures of purified bone marrow progenitors (*46*). In these experiments, IL-3 proved to be much more effective than GM-CSF at supporting 21-day colony formation by the normal human blasts, and the resulting colonies could be replated to yield secondary colonies with much higher efficiency than was attained with the GM-CSF–derived colonies. Many of the IL-3–supported secondary colonies were found to contain multiple cell lineages, demonstrating that the gibbon IL-3 will support proliferation by early human hematopoietic blast cells that retain at least some self-renewal capacity. These results clearly establish a population of hematopoietic target cells for IL-3 that are significantly earlier and more pluripotent than any GM-CSF–responsive cell.

The biological activity of the human M-CSF remains something of an enigma. While either form of human M-CSF is a potent stimulator of murine macrophage colony formation, both forms are much less active with human target cells in the culture systems studied so far (*16, 18*). Whether this is due to difficulty in growing human macrophages in vitro or represents the real biological activity of M-CSF remains to be seen. M-CSF from natural sources is capable of activating mature macrophages (*53*), and it is possible that the human M-CSF functions as a survival and activating factor for monocytes and macrophages. It is encouraging in this regard that the larger form of recombinant M-CSF is a potent potentiator of human macrophage cytotoxicity (*54*). It will be of great interest to compare the activities of the two different forms of M-CSF to see if they might mediate different effects associated with this factor.

Relatively little is known about the receptors for the human CSFs, with the exception of those for the M-CSF. Two groups have reported the detection of very low numbers of receptors for G- and GM-CSF on myeloid cells (*55, 56*). Cross-linking studies have suggested that the G-CSF receptor may correspond to a 30-kD polypeptide (*55*). Receptors for M-CSF are, by contrast, relatively more abundant on cells of the monocytic lineage (*57*). The recent identification of the M-CSF receptor as the gene product of the c-*fms* proto-oncogene has generated considerable excitement (*27*). The availability of pure recombinant CSFs and their genes will undoubtedly result in considerable progress in the receptor area in the near future.

Activities of the CSFs in Vivo

One of the major consequences of the cloning of the CSF genes has been the availability of large quantities of highly purified protein for animal experiments. Although results from these studies are frequently difficult to interpret, they do provide insight into the function of the CSFs that is not obtainable from in vitro studies. Thus far, primate studies have been completed with the recombinant human G-CSFs (*39*) and GM-CSFs (*36, 58*). Murine IL-3 has also been tested in mice (*59*), and GM-CSF has been studied in man (*60*).

Initial experiments with human GM-CSF (*58*) demonstrated that continuous intravenous infusion of human GM-CSF into normal monkeys (*Macaca fasicularis* and *M. mulatta*) elicited a dramatic leukocytosis in a dose-dependent fashion. Total white cell counts of 50,000 or greater (8,000 to 10,000 per cubic millimeter is normal in *M. fasicularis*) could be achieved within 24 to 48 hours, and these levels were maintained for as long as 1 month by continuing the infusion. In all cases, the total white cell count returned to normal within 3 to 7 days after termination of treatment. Monkeys remained afebrile and showed no induction of toxic side effects. The elevated cell counts resulted largely from increased numbers of neutrophils, banded neutrophils, and eosinophils. When assayed in vitro, neutrophils from such monkeys were functionally primed. Usually the animals also had high levels of circulating monocytes and lymphocytes, although these effects have been somewhat variable. In one severely pancytopenic animal, GM-CSF restored white blood cell levels to well above normal and also induced a marked reticulocytosis. More recent experiments (*61*) showed that administration of GM-CSF enhanced the recovery of neutrophils in monkeys undergoing autologous bone marrow transplantation. In these experiments, the neutrophils in treated animals reached a low normal level as much as 7 days earlier than in controls. However, the observed elevation was transient and depended on continued infusion with GM-CSF. This suggests an effect on later progenitor cells in the graft and not on early progenitors capable of repopulating the bone marrow. A similar effect on platelet count was observed, consistent with in vitro studies showing that GM-CSF will support the proliferation of some megakaryocyte progenitors, although it should be remembered that it is not possible to distinguish direct and indirect effects in animal models.

More recently, recombinant GM-CSF that we produced from genetically engineered Chinese hamster ovary cells was administered to neutropenic patients with acquired immune deficiency syndrome (AIDS). This was part of the first-phase I/II clinical trial with a colony-stimulating factor. The clinical investigators reported a dose-dependent increase in white blood cell count that corrected the neutropenia and achieved leukocytosis at the higher doses (*60*). In general, the responses were those predicted by the experiments on lower primates (*36, 58*), although the effective human dosages were somewhat lower than those necessary in monkeys. The GM-CSF caused no adverse reactions or symptoms that could be distinguished from those normally occurring in AIDS patients.

Administration of recombinant human G-CSF to normal monkeys (twice daily subcutaneous injection) elicited a similar elevation in white count which was also dependent on continued administration of the hematopoietin; no significant elevations in the monocyte, eosinophil, or reticulocyte counts were observed, although the numbers of circulating T cells were increased (*39*). Analysis of the neutrophils in vitro demonstrated that they were functional and had enhanced ability to kill and phagocytize bacteria. Even more dramatic results have been achieved in monkeys treated with doses of cyclophosphamide sufficient to cause bone marrow aplasia and pancytopenia. Typically, such animals require 4 weeks or longer before the white cell counts return to normal. Cyclophosphamide-treated monkeys that received G-CSF had normal or elevated white cell counts by 7 days after the bone marrow oblation. In this case, the white cell counts returned to normal after cessation of the G-CSF administration. Although the increased numbers of circulating cells were mainly neutrophils, analysis of bone marrow aspirates in the treated monkeys indicated that the recovery of all of the lineages was accelerated relative to control animals.

Therapeutic Application of CSFs

The medical applications for colony-stimulating factors lie in three general areas: restoration of hematopoietic dysfunction by raising cell counts from suppressed to normal levels; augmentation of host defense against infection; and, possibly, in malignant disease, stimulating the hyperproduction of functionally primed effector cells (*62*).

The correction of blood cell deficiencies is the most conservative of the three therapeutic expectations. Suppression of myelopoiesis resulting in granulocytopenia, thrombocytopenia, and anemia is a frequent side effect of chemotherapy or radiation therapy for cancer. Indeed, such hematopoietic dysfunction is the major cause of morbidity and mortality in cancer patients (*63*). Treatment of

rheumatoid arthritis and other autoimmune disorders with cytotoxic drugs is limited by bone marrow toxicity. Severe burn victims commonly also have extremely low white blood cell levels. Other disorders of bone marrow function, especially the pancytopenia often associated with AIDS and exaggerated by treatment with myelosuppressive drugs, are of major medical concern today (*64*). While anemia and thrombocytopenia can often be treated by transfusion, there is no effective method to restore granulocyte and monocyte-macrophage levels. As granulocytes are extremely short-lived in the circulation, the major clinical problem arises from deficiency in neutrophilic granulocytes. Such neutropenic patients are extremely susceptible to infection by bacteria, fungi, and other parasites. The dramatic evidence demonstrating the ability of GM-CSF to regulate in vivo granulocyte levels in primates (*58*) thus encouraged Sandoz to initiate clinical evaluation of recombinant GM-CSF produced by Genetics Institute for the treatment of neutropenia induced by a variety of causes from chemotherapy to bone marrow transplantation and AIDS. Similarly, Amgen has begun to study recombinant G-CSF in cancer patients. The successful outcome of the AIDS study (*60*) demonstrated for the first time that a CSF can be safely used to elevate white blood cell counts in man. Other groups (Amgen, Immunex, Schering-Plough) have begun or are planning clinical studies with G- or GM-CSF. Clinicians are highly encouraged by the success with GM-CSF, and that of the Amgen-sponsored trial demonstrating the effectiveness of another hematopoietic growth factor, erythropoietin, in the treatment of anemia due to end-stage renal disease (*65*).

All of the CSFs share the property of being potentiators which increase the responsiveness of mature effector cells when they are subsequently exposed to an appropriate activating stimulus (*44, 47*). Leukocytes—both granulocytes and cells of the monocyte-macrophage family—are relatively ineffective in killing and phagocytosis unless they are activated by one or more of a variety of agents involved in the inflammatory response. The cytokines IL-1 and immune interferon are important in this process but, surprisingly, the CSFs—originally thought to be primarily growth factors—have all proved to have the additional ability to enhance the sensitivity of phagocytes to their natural triggering substances, including bacterial peptides, lipopolysacchrides, and opsonized particles. Many hematologists are now hopeful that boosting host defense against infection and malignancy will become the most common applications of CSFs. GM-CSF and G-CSF could be useful in bacterial infection, where neutrophil responses are most significant. M-CSF, as well as GM-CSF, will be considered for macrophage-mediated disease defense mechanisms against intracellular pathogenic bacteria and fungi, as well as other intracellular parasites; such infections are common in AIDS patients. Augmentation of macrophage number and function with M-CSF and GM-CSF also will be tested as a treatment for certain forms of cancer, perhaps in combination with antitumor monoclonal antibodies to mediate antibody-dependent cellular cytotoxicity.

The thrombocytopenia associated with bone marrow suppression may also be treatable by CSF therapy. Although GM-CSF has not thus far elevated platelet levels in primates or humans with normal platelet levels, four out of five rhesus monkeys treated with GM-CSF subsequent to autologous bone marrow transplantation showed accelerated platelet recoveries (*58*). Because IL-3 is an effective megakaryocyte CSF in vitro, it will be interesting to determine whether it functions as a platelet regulator in vivo.

Combinations of different CSFs may eventually prove most efficacious. For example, severe anemia may respond well to the combination of GM-CSF or IL-3 (both of which have erythroid burst–promoting activity) with erythropoietin. IL-3 could be used with GM-CSF to decrease the time required to recover from bone marrow transplantation, or with IL-2 to stimulate lymphocyte production. Use of either GM-CSF or IL-3 with G-CSF might be expected to maximize granulocyte responses.

It is premature to predict what role, if any, CSFs could play in leukemia therapy. Myeloid leukemic cells can use IL-3, GM-CSF, and G-CSF as growth factors (*66*). While some might argue that there could be therapeutic opportunities afforded by increasing the proliferative rate of CSF-responsive malignant cells, thus making them more sensitive to chemotherapy, it is equally apparent that such treatment would not be without risk. Similarly, selected patients may benefit from G-CSF therapy if their leukemic cells respond to the factor by rapid terminal differentiation. However, in these patients as well, this therapy could prove harmful if the clonogenic leukemic cells, which usually represent a small proportion of the total cell population, proliferate in response to exposure to G-CSF. Lymphoid leukemias, however, do not respond to GM- or G-CSF, and thus colony-stimulating factors may find a role in the treatment of these diseases and lymphomas.

Systemic administration of any potent immunomodulator might be expected to elicit significant toxic side effects. Hopes for the use of agents like TNF, IL-1, lymphotoxin, and IL-2 have been partially compromised by moderate to severe toxicity in patients. Thus far, the preclinical studies done with GM-CSF (*58*) and G-CSF (*39*) in primates have demonstrated remarkable safety. Initial clinical results are entirely consistent with these predictions (*60*). Animals have been maintained with extremely high white blood cell counts with no significant adverse effect and no toxicity. This may be because primed phagocytes become activated only when triggered by stimuli that do not occur in normal animals. Thus, CSFs can potentially be used to deliver large numbers of primed effector cells to sites of infection or tumors, which should augment the local release of cytotoxic agents including IL-1, TNF, and interferons. This concept warrants careful clinical evaluation to determine whether it affords a beneficial therapeutic index compared with systemic administration of the cytotoxic proteins themselves. Because of the availability of the recombinant CSFs, these concepts are being tested. We expect the next review of the human CSFs in *Science* to be focused on the utility of these interesting molecules in treating human disease.

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Regulation of Inducible and Tissue-Specific Gene Expression

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Molecular genetics approaches have been used to identify and characterize cis-acting DNA sequences required for eukaryotic gene regulation. These sequences are modular in nature, consisting of arrays of short (10– to 12–base pair) recognition elements that interact with specific transcription factors. Some transcription factors have been extensively purified and the corresponding genes have been cloned, but the mechanisms by which they promote transcription are not yet understood. Positive and negative regulatory elements that function only in specific cell types or in response to extracellular inducers have been identified. A number of cases of inducible and tissue-specific gene expression involve the activation of preexisting transcription factors, rather than the synthesis of new proteins. This activation may involve covalent modification of the protein or an allosteric change in its structure. The modification of regulatory proteins may play a central role in the mechanisms of eukaryotic gene regulation.

A CENTRAL PROBLEM IN EUKARYOTIC MOLECULAR BIOLOGY is to understand the mechanisms by which specific genes are expressed in a temporal or tissue-specific manner or are activated in response to extracellular inducers. The development of methods for cloning and characterizing individual genes has provided the opportunity to study these mechanisms at the molecular level. Initially, cis-acting DNA sequences required for gene regulation were identified by introducing mutations into cloned genes and then analyzing their effects on expression in vivo. More recently, proteins that specifically bind to these regulatory DNA sequences have been identified and in some cases purified. The current challenges are to understand how specific protein-DNA interactions regulate gene expression and how these interactions are integrated into the overall pattern of gene regulation during development. In this review, we summarize the information obtained to date regarding the nature of the DNA sequence elements and protein factors required for gene regulation at the level of transcription initiation in higher eukary-

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