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(57) Abstract

Compositions are provided, including nucleic acids, polypeptides, and antibodies, that can be used to regulate hematopoietic progenitor cell development or differentiation. Methods for using these materials, as well as kits for quantifying the materials, are also provided.

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MODULATORS OF HEMATOPOIETIC PROGENITOR CELLS

The present invention relates to methods and compositions for modulating the development of cells in a mammalian circulatory system. More particularly, this invention relates to particular compositions and their uses in methods to regulate development of blood cells.

BACKGROUND OF THE INVENTION

The circulating component of the mammalian circulatory system is composed of various cell types, including red and white blood cells of the erythroid or the myeloid cell lineages. See, e.g., Rapaport, *Introduction to Hematology* (2d ed.), 1987, Lippincott, Philadelphia, PA; and Jandl, *Blood: Textbook of Hematology*, 1987, Little, Brown and Co., Boston, MA. Red blood or erythroid cell production occurs through the committed erythroid progenitor cell lineage and is stimulated in the terminal stages of differentiation by the protein erythropoietin (EPO).

The bone marrow contains a rare population of primitive cells, pluripotent hematopoietic progenitor cells, e.g., stem cells, that have the capacity to differentiate into any mature cell in the peripheral circulation. The stem cells can either proliferate and generate cells with nearly the identical capacity (self-renewal) or start the differentiation pathway of becoming more restricted in the production of a given cell type, eventually having the ability of generating one type of cell. Cells committed to the red blood cell, or erythroid, lineage have an indistinctive morphology and can only be identified indirectly using specific *in vitro* assays that evaluate the resulting progeny cell. See Metcalf, *The Hematopoietic Colony Stimulating Factors*, 1984, Elsevier, NY.

The immediate precursors of erythropoietin-sensitive progenitors are of particular value because they serve as a reserve for differentiation to mature red blood cells (RBC) when necessary. Such needs may arise from blood loss, anemia, or similar problems, e.g., as a result of chemo- or radiation-therapy. Alternatively, conditions of excessive hematopoietic cell production, e.g., hematopoietic cell proliferative disorders, may result from abnormal regulation by factors which promote cellular development.

Many factors have been identified in the differentiation process of these cells, including the cytokines IL-3, IL-4, GM-CSF, IL-9, and SCF. See, e.g., Zsebo *et al., Cell 63*:195 (1990); and Migliacio *et al., J. Cell Physiol. 148*:503 (1991). These cytokines stimulate early stages of erythroid differentiation *in vitro*, but only the latter stimulate red blood cell differentiation *in vivo*.

However, other factors may exist whose functions in hematopoiesis were heretofore unrecognized. These factors should provide for biological activities whose spectra of effects provide advantages over known differentiation factors. There thus is a need to identify more of these other factors.

SUMMARY OF THE INVENTION

The present invention fills this need by providing new classes of hematopoietic progenitor cell differentiation factors designated hematopoietic regulators (HR), the biological effects of which have been unrecognized. In particular, two new classes of hematopoietic regulator polypeptides, exemplified by HR1 and HR2, are described herein.

The HR1 class of polypeptides are characterized by structural features of having sequence homology to a sequence of defined characteristics or biological activity of stimulating hematopoietic development, and is exemplified

herein by a mouse protein designated mouse HR1 (mHR1), or the 38 kD protein.

The HR2 class of polypeptides is characterized by structural features of having sequence homology to a defined sequence or biological activity of inhibiting hematopoietic development, and is exemplified herein by a mouse protein designated mouse HR2 (mHR2), or the 18 kD protein.

The present invention provides an isolated or recombinant nucleic acid comprising sequence from a member of a class of hematopoietic regulatory factor, which comprises an amino acid sequence homologous to an HR1 polypeptide or an HR2 polypeptide. In one embodiment, the sequence is homologous to a sequence from an HR1 polypeptide, and the polypeptide is capable of stimulating differentiation of a hematopoietic progenitor cell, e.g., a bone-marrow-derived progenitor cell, a mast cell, or a myeloid cell. In a second embodiment, the sequence is homologous to a sequence from an HR2 polypeptide, and the polypeptide is capable of inhibiting differentiation of a hematopoietic progenitor cell.

The present invention further provides a recombinant polypeptide capable of regulating hematopoietic progenitor cell development, wherein the polypeptide comprises an amino acid sequence homologous to an HR1 polypeptide or an HR2 polypeptide. In one embodiment, the polypeptide stimulates development of a hematopoietic progenitor cell and the sequence is selected from an HR1 polypeptide. In an alternative embodiment, the polypeptide inhibits development of a hematopoietic progenitor cell and the sequence is selected from an HR2 polypeptide.

This invention also provides an antibody which recognizes an HR1 polypeptide or an HR2 polypeptide.

Other embodiments provide methods of modulating development of a hematopoietic progenitor cell comprising a step of contacting the cell with a compound selected from an HR1 polypeptide or analog, an antagonist of an HR1

polypeptide, an HR2 polypeptide or analog, or an antagonist of an HR2 polypeptide. In one such embodiment, the compound is an HR1 polypeptide, and the polypeptide stimulates differentiation of a hematopoietic progenitor cell. In another embodiment, the compound is an HR2 polypeptide, and the polypeptide inhibits differentiation of a hematopoietic progenitor cell.

The present invention also provides kits for determining the amount, in a sample, of a regulatory factor for hematopoietic progenitor cells, comprising at least one reagent selected from a nucleic acid capable of hybridizing under stringent hybridization conditions with one encoding an HR1 polypeptide; an HR1 polypeptide or analog; an antibody specific for an HR1 polypeptide or analog; a nucleic acid capable of hybridizing under stringent hybridization conditions with one encoding an HR2 polypeptide; an HR2 polypeptide or analog; or an antibody specific for an HR2 polypeptide or analog.

DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

General

Red blood cell (RBC) production is regulated at the terminal stages of differentiation primarily by erythropoietin (EPO). Macrophages secrete EPO and probably supply EPO to erythroid progenitors in the bone marrow. In states of erythropoietic stress, such as massive blood loss or hypoxia, EPO is produced in the kidney by a highly regulated oxygen sensing mechanism. Exogenous infusion of recombinant EPO significantly boosts RBC production in people suffering from anemia caused by renal failure, chemotherapy, or the HIV virus. The increase in RBC's, however, requires the continuous

treatment with EPO-RBC levels return to previous baselines within a day upon termination of EPO therapy. Using regulators that act on progenitors committed to the erythroid lineage but not yet responsive to EPO might better sustain RBC production and help patients suffering from bone marrow failure.

Some cytokines such as IL-3, IL-4, GM-CSF and IL-9 have been known to act *in vitro* to stimulate primitive erythroid progenitors and to augment the action of EPO. However, RBC levels do not significantly increase in people treated with either IL-3 or GM-CSF. Mice lacking the IL-4 gene, inactivated by homologous recombination, are not noticeably anemic. The role of these cytokines *in vivo* may be indirect but probably are not of primary importance.

The strongest evidence for a physiologically relevant regulator comes from the recently identified ligand for the c-kit proto-oncogene, kit ligand (KL) or stem cell factor (SCF). This factor stimulates *in vitro* both primitive erythroid progenitors, as evaluated by burst forming units-erythroid (BFU-E), and mature erythroid progenitors, as evaluated by colony forming units-erythroid (CFU-E), and boosts RBC and other cell types when infused in mice. A homozygous mutation in mice of either the kit ligand (Sl/Sl) or its receptor (W/W) is lethal. Mice with viable forms of these mutations suffer from anemia, which for Sl/Sl mice can be reversed by infusion of the normal kit ligand.

Erythroid potentiating activity (EPA), a protein that is identical to tissue inhibitor of metalloproteases (TIMP), was identified in a T cell source by its ability to stimulate erythropoiesis *in vitro*; injection of EPA in mice increases erythroid progenitor frequency and RBC's.

In an effort to find more physiologic regulators of erythropoiesis, the unique properties of erythroid progenitors were used to develop a rapid assay to identify novel erythroid regulators. This assay was rapid, allowing screening of large

numbers of samples, and sensitive. The actions of known cytokines were determined in the assay. A variety of supernatants from hematopoietic and non-hematopoietic cell sources were tested for their capacity to stimulate erythropoiesis *in vitro*. A T cell source was selected, and large batches were prepared for biochemical purification.

Two activities have been purified to near homogeneity and subjected to N-terminal sequence analysis by Edman degradation. Both were blocked at the amino terminus, and no sequence data was obtained. One activity corresponded to an inhibitory activity for CFU-E, and this activity was purified. Further analysis of cyanogen bromide fragments of the protein corresponding to this activity revealed a partial amino acid sequence that shares high homology with the wnt family of proteins. Pursuit of wnt-type proteins found in hematopoietic cDNA libraries has revealed the presence of at least one new protein of the class, designated HR2. This is the first report of the expression of wnt-like proteins, and the first indication of any activity for such proteins, e.g., modulation of hematopoiesis.

A hematopoietic-specific, erythroid-specific, or myeloid-specific sequence is one which is found in such cell lineages, but has not heretofore been localized to expression in the respective hematopoietic cell lineages. Thus, specific sequences need not be found exclusively within the respective hematopoietic, erythroid, or myeloid cell lineage.

Agonists or antagonists of the hematopoietic regulatory factor activity are molecules which functionally operate to simulate or block the modulatory function of the respective factor. Generally, the terms agonist or analog do not exclude the natural or recombinantly produced factors having natural primary polypeptide sequences.

HR1 Class Proteins

Nucleic Acids

The present invention provides novel nucleic acids encoding proteins or fragments thereof which exhibit the described biological activities. It also provides the means and reagents useful to fully clone and characterize the natural genes which encode this biological activity. The present disclosure teaches methods for purification of the protein from natural sources, thereby enabling production of quantities of protein allowing sequence characterization. This will allow isolation without undue difficulty of the natural gene encoding the protein using techniques described, e.g., in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2d ed.), 1989, Cold Spring Harbor Press, CSH, NY; Ausubel *et al.* (1987 and periodic supplements), *Current Protocols in Molecular Biology*, Greene/Wiley, NY; and Davis et al., *Basic Methods in Molecular Biology*, 1986, Elsevier Science Publishing Co., NY.

The present invention further encompasses recombinant DNA molecules and fragments having a DNA sequence identical to or highly homologous to DNAs described herein, or whose purification is derived from these data. In particular, the sequences will often be operably linked to DNA segments which control transcription, translation, and DNA replication.

Homologous nucleic acid sequences, when compared, exhibit significant similarity. The standards for homology in nucleic acids are either measures for homology generally used in the art by sequence comparison or based upon hybridization conditions. The hybridization conditions are described in greater detail below. Homology measures for the mHR1 protein are further limited by the homology to either of the gCap39 or Mbh1 proteins. See Yu *et al.*, *Science 250*:1413 (1990) (gCap39); and Prendergast *et al.*, *EMBO J. 10*:757 (1991) (Mbh1). Homology measures will be limited, in

addition to any stated parameters, to exceed any such similarity to the genes encoding these two proteins.

Substantial nucleic acid sequence homology means either that the segments, or their complementary strands, when compared, are identical when optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 50% of the nucleotides, preferably in at least 74%, more preferably in at least 90% and most preferably in at least about 95% of the nucleotides.

Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to a strand, or its complement, using a sequence described. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. See, Kanehisa, *Nucleic Acids Res. 12*:203 (1984).

The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will be over a stretch of at least about 17 nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at least about 28 nucleotides, more typically at least about 40 nucleotides, preferably at least about 50 nucleotides, and more preferably at least about 75 to 100 or more nucleotides.

Stringent conditions, in referring to homology in the hybridization context, will be stringent combined conditions of salt, temperature, organic solvents, and other parameters typically controlled in hybridization reactions. Stringent temperature conditions will usually include temperatures in excess of about 30° C, more usually in excess of about 37° C, typically in excess of about 45° C, more typically in excess of about 55° C, preferably in excess of about 65° C, and more preferably in excess of about 70° C.

Stringent salt conditions will ordinarily be less than about 1000 mM, usually less than about 500 mM, more usually less than about 400 mM, typically less than about 300 mM, preferably less than about 200 mM, and more preferably less than about 150 mM. However, the combination of parameters is much more important than the measure of any single parameter. See, e.g., Wetmur and Davidson, *J. Mol. Biol.* *31*:349 (1968).

Proteins

The present invention provides natural proteins, and allelic and natural variants thereof which exhibit the described modulatory effects on hematopoietic cells, especially erythroid hematopoietic cell types and lineages. In one embodiment, the HR1 class of regulatory factors, an activity has been isolated corresponding to a 38 kD protein, and portions of its sequence have been determined.

The amino acid sequences of these parts of the 38 kD protein, presented in order of appearance within the complete protein, from amino to carboxyl terminus, are defined in the Sequence Listing by SEQ ID NOs 1-9.

The present invention also provides proteins or peptides having substantial amino acid sequence homology with the amino acid sequences defined by SEQ ID NOs 1-9, but excluding any protein or peptide which exhibits substantially the same or lesser amino acid sequence homology than does gCap39 or Mbh1. Other variants are also provided which are distinguishable from natural protein, including either soluble forms or cell bound forms, along with variants possessing non-primary structure modifications, e.g., glycosylation patterns, acetylations, and carboxylations.

A polypeptide "fragment", or "segment", is a stretch of amino acid residues of at least about 8 amino acids, generally at least 10 amino acids, more generally at least 12 amino acids, often at least 14 amino acids, more often at least 16

amino acids, typically at least 18 amino acids, more typically at least 20 amino acids, usually at least 22 amino acids, more usually at least 24 amino acids, preferably at least 26 amino acids, more preferably at least 28 amino acids, and, in particularly preferred embodiments, at least about 30 or more amino acids.

Amino acid sequence homology, or sequence identity, is determined by optimizing residue matches in primary sequence, if necessary, by introducing gaps as required. This changes when considering conservative substitutions as matches. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Homologous amino acid sequences are intended to include natural allelic and interspecies variations in each respective regulatory factor sequence.

Typical homologous proteins or peptides will have from 75-100% homology (if gaps can be introduced), to 90-100% homology (if conservative substitutions are included) with the amino acid sequences defined by SEQ ID NOs 1-9. Homology measures will be at least about 60%, generally at least 70%, more generally at least 75%, often at least 80%, more often at least 85%, typically at least 88%, more typically at least 91%, usually at least 93%, more usually at least 95%, preferably at least 97%, and more preferably at least 98%, and in particularly preferred embodiments, at least 99% or more. Some homologous proteins or peptides, such as allelic or species variants of mHR1, will share various biological activities with the factor, the amino acid sequences of which are defined by SEQ ID NOs 1-9.

Biological Activities

Supernatants from ConA stimulated T cells were used as a source to assay for colony stimulating factor-erythroid (CFU-E) activity. Batches of medium from the T cell line D10.G4.1, were generated [Kaye *et al., J. Exptl Med. 158*:836 (1983). Known cytokines (IL-3, IL-4, IL-5, IL-6, and GM-CSF were removed by antibody affinity columns. The CFU-E activities from the supernatants were purified through a series of biochemical purification steps, including hydroxylapatite, hydrophobic interaction, gel filtration, and anion exchange or reverse phase column chromatography steps. The purification process monitored modulatory activity on murine hematopoietic progenitors, including enriched bone marrow-derived progenitors (lin^-,SCA^- phenotype), a mast cell line MC9, and a myeloid cell line (DA1.A). The defined method for purification provides sufficient physical characteristics which allow identification and distinguishing of this embodiment from other hematopoietic regulatory factors.

The isolated factor has defined activities on crude bone marrow cultures and purified progenitor cell populations, and its biological effects on other aspects of hematopoietic cell development will be easily tested by standard or modified tests for regulatory function. Specifically, the *in vivo* actions of these proteins will be tested taking standard approaches, e.g., injection of purified protein or antibodies thereto either into neonatal animals to test the effect on development, or into animals placed under hematopoietic stress by massive blood loss, hypoxia, radiation treatment, infection by viruses or microorganisms, or other disease models; or investigating effects on development of transgenic models where the gene encoding the HR1 or HR2 protein is over- or underexpressed; or modulating expression of genes encoding HR1 or HR2 in mice using homologous recombination techniques. See, e.g., Coligan *et al.* (1991 and

periodic supplements), *Current Protocols in Immunology*, Greene/Wiley, NY.

The mHR1 embodiment of the HR1 class of regulatory factors is characterized by the amino acid sequences defined by SEQ ID NOs 1-9. In particular, the protein appears to be related to a family of proteins related to gelsolin. Although the protein exhibits substantial homology to two other proteins designated gCap39 and Mbh1, it has a number of structural differences. See Yu *et al.*, *Science*, and Prendergast *et al.*, *EMBO J.*

HR2 Class Proteins

Nucleic Acids

The present invention provides synthetic nucleic acids encoding proteins described herein, particularly encoding the amino acid sequence disclosed in Table 1. Also, natural nucleic acid sequences which encode HR2 proteins are provided. Homology relative to mHR2 is similar to that described above for mHR1, but is limited by homology to other int/wnt related nucleic acid sequences derived from non-hematopoietic cDNA libraries. See, e.g., Nusse *et al.*, *Cell 69*:1073 (1992).

Table 1

protein sequence LFGKIVNR

wnt sequences
Y = pyrimidines
R = purines
Primer sequences (degenerate):

Wnt.1+ GATCGCGGCCGCCARGARTGYAARTGYCAT (forward)
Wnt.1- GTACCCGCGGRCARCACCARTGRAA (reverse)

[protein sequence from Gavin *et al., Genes & Development* *4*:2319 (1990), Figure 2]

WntX clone #32:

CATGG CACAT CAGGC AGCTG CCAGT TCAAG ACATG CTGGA GGGCG
GCCCC AGAGT TCCGG GCAGT GGGGG CGGCG TTGAG GGAGC GGCTG
GGCCG GGCCA TCTTC ATTGA TACCC ACAAC CGCAA TTCTG GAGCC
TTCCA GCCCC GTCTG CGTCC CCGTC GCCTC TCAGG AGAGC TGGTC
TACTT TGAGA AGTCT CCTGA CTTCT GTGAG CGAGA C

The nucleotide and amino acid sequences of a wnt DNA and protein (HR2 polypeptide) are defined in the Sequence Listing by SEQ ID NOs 16 and 17, respectively.

Proteins

Concepts describing the HR1 class of proteins are similarly applicable to HR2 proteins, with the further limitations of the gCap39 and Mbh1 exclusions substituted by int/wnt proteins described above. The HR2 class of proteins is exemplified by one 18 kD protein which has been characterized. Using PCR techniques to screen for related cDNA molecules from hematopoietic cells, one related transcript was isolated. The expected inhibitory activity on

hematopoietic progenitor cell development is found in the expression products of this cDNA molecule. Again, variants of the described proteins are also encompassed within the present invention, including new proteins having soluble or membrane bound properties.

Biological Activities

The biological activities described for the HR1 class of proteins are similar for the HR2 class, but the HR2 class have an inhibitory effect. Further testing of the protein for additional activities will be performed as described above for the HR1 class. See, e.g., Coligan *et al.*, *Current Protocols in Immunology*.

Making Proteins; Analogues

Natural Isolation

The present invention provides means for isolating members of the classes of HR1 and HR2 proteins. In particular, methods are described for purifying the HR1 embodiment by its physical characteristics, particularly those protein chemistry features described. Specific purification techniques are described, and modifications can be made as appropriate. Moreover, since the present invention provides isolated protein, it can be further characterized to provide new methods for isolation. For example, new affinity techniques may be applied using antibody or other binding molecules.

Likewise, with the HR2 protein and related peptides, Similar methods were applied, with the distinguishing physical characteristics as described. Many of the applicable methods for handling the regulatory factors will be shared, though the specific conditions and parameter leading to purification of each factor will be distinct.

In particular, analogues of each factor are provided, including post-translational chemical modification of natural or recombinant forms, which may confer desirable physiological or biological properties, e.g., modified half-life, absorption characteristics, membrane association, etc.

Synthetic Methods

The regulatory factors, fragments, or derivatives thereof can be prepared by conventional processes for chemically synthesizing peptides. These include processes such as are described in Stewart and Young, *Solid Phase Peptide Synthesis*, 1984, Pierce Chemical Co., Rockford, IL; Bodanszky and Bodanszky, *The Practice of Peptide Synthesis*, 1984, Springer-Verlag, New York; Bodanszky, *The Principles of Peptide Synthesis*, 1984, Springer-Verlag, New York; Merrifield, *J. Amer. Chem. Soc. 85*:2149 (1963); Merrifield, *Science 232*: 341 (1986); and Atherton *et al., Solid Phase Peptide Synthesis: A Practical Approach*, 1989, IRL Press, Oxford.

For example, an azide process, an acid chloride process, an acid anhydride process, a mixed anhydride process, an active ester process (for example, p-nitrophenyl ester, N-hydroxysuccinimide ester, or cyanomethyl ester), a carbodiimidazole process, an oxidative-reductive process, or a dicyclohexylcarbodiimide (DCCD)/additive process can be used. Solid phase and solution phase syntheses are both applicable to the foregoing processes.

These regulatory factors, fragments or derivatives are suitably prepared in accordance with the above processes as typically employed in peptide synthesis, generally either by a so-called stepwise process which comprises condensing an amino acid to the terminal amino acid, one by one in sequence, or by coupling peptide fragments to the terminal amino acid. Amino groups that are not being used in the coupling reaction must be protected to prevent coupling at an incorrect location.

If a solid phase synthesis is adopted, the C-terminal amino acid is typically bound to an insoluble carrier or support through its carboxyl group. The insoluble carrier is not particularly limited as long as it has a binding capability to a reactive carboxyl group. Examples of such insoluble carriers include halomethyl resins, such as chloromethyl resin or bromomethyl resin, hydroxymethyl resins, phenol resins, tert-alkyloxy carbonylhydrazidated resins, and the like.

An amino group-protected amino acid is bound in sequence through condensation of its activated carboxyl group and the reactive amino group of the previously formed peptide or chain, to synthesize the peptide step by step. After synthesizing the complete sequence, the peptide is split off from the insoluble carrier to produce the peptide. This solid-phase approach is generally described by Merrifield *et al., J. Am. Chem. Soc. 85*:2149 (1963).

The prepared regulatory factor and fragments thereof can be isolated and purified from the reaction mixture by means of peptide separation, e.g., by extraction, precipitation, electrophoresis, and various forms of chromatography, and the like. The factors of this invention can be obtained in varying degrees of purity depending upon its desired use. Purification can be accomplished by use of the protein purification techniques disclosed herein or by the use of the antibodies herein described in immuno-absorbent affinity chromatography. This immuno-absorbent affinity chromatography is carried out by first linking the antibodies to a solid support and then contacting the linked antibodies with solubilized lysates of appropriate cells, lysates of other cells expressing the regulatory factor, or lysates or supernatants of cells producing them as a result of DNA techniques, see below. Combinations of recombinant techniques with removable fusion segments which allow for simple purification are also available.

Recombinant Methods

DNA which encodes these regulatory factors or fragments thereof can be obtained by chemical synthesis, screening cDNA libraries, or by screening genomic libraries prepared from a wide variety of cell lines or tissue samples.

This DNA can be expressed in a wide variety of host cells for the synthesis of a full-length regulatory factor or fragments of a factor which can in turn, for example, be used to generate polyclonal or monoclonal antibodies; for binding studies; for construction and expression of modified molecules; and for structure/function studies. Each factor or its fragments can be expressed in host cells that are transformed or transfected with appropriate expression vectors. These molecules can be substantially free of protein or cellular contaminants, other than those derived from the recombinant host, and therefore are particularly useful in pharmaceutical compositions when combined with a pharmaceutically acceptable carrier and/or diluent. The factor, or portions thereof, may be expressed as fusions with other proteins, often useful for purification schemes.

Expression vectors are typically self-replicating DNA or RNA constructs containing the desired gene or its fragments, usually operably linked to suitable genetic control elements that are recognized in a suitable host cell. These control elements are capable of effecting expression within a suitable host. The specific type of control elements necessary to effect expression will depend upon the eventual host cell used. Generally, the genetic control elements can include a prokaryotic promoter system or a eukaryotic promoter expression control system, and typically include a transcriptional promoter, an optional operator to control the onset of transcription, transcription enhancers to elevate the level of mRNA expression, a sequence that encodes a suitable ribosome binding site, and sequences that terminate transcription and translation. Expression vectors also usually

contain an origin of replication that allows the vector to replicate independently of the host cell.

The vectors of this invention contain DNA which encodes a described regulatory factor, or a fragment thereof, typically encoding a biologically active polypeptide. The DNA can be under the control of a viral promoter and can encode a selection marker. This invention further contemplates use of such expression vectors which are capable of expressing eukaryotic cDNA coding for a regulatory factor in a prokaryotic or eukaryotic host, where the vector is compatible with the host and where the eukaryotic cDNA coding for the factor is inserted into the vector such that growth of the host containing the vector expresses the cDNA in question. Usually, expression vectors are designed for stable replication in their host cells or for amplification to greatly increase the total number of copies of the desirable gene per cell. It is not always necessary to require that an expression vector replicate in a host cell, e.g., it is possible to effect transient expression of the desired factor or its fragments in various hosts using vectors that do not contain a replication origin that is recognized by the host cell. It is also possible to use vectors that cause integration of a gene encoding a factor or its fragments into the host DNA by recombination.

Vectors, as used herein, comprise plasmids, viruses, bacteriophage, integratable DNA fragments, and other vehicles which enable the integration of DNA fragments into the genome of the host. Expression vectors are specialized vectors which contain genetic control elements that effect expression of operably linked genes. Plasmids are the most commonly used form of vector but all other forms of vectors which serve an equivalent function and which are, or become, known in the art are suitable for use herein. See, e.g., Pouwels *et al.* (1985 and Supplements), *Cloning Vectors: A Laboratory Manual*, Elsevier, NY; and Rodriquez *et al.* (eds), *Vectors: A*

Survey of Molecular Cloning Vectors and Their Uses, 1988, Buttersworth, Boston.

Transformed cells are cells, preferably mammalian, that have been transformed or transfected, e.g., with the appropriate vectors constructed using recombinant DNA techniques. Transformed host cells usually express the desired factor or its fragments, but for purposes of cloning, amplifying, and manipulating its DNA, do not need to express the protein. This invention further contemplates culturing transformed cells in a nutrient medium, thus permitting the factor to accumulate in the culture, whether extracellular or otherwise, and soluble or otherwise. The regulatory factor can be recovered, either from the culture or from the culture medium.

For purposes of this invention, DNA sequences are operably linked when they are functionally related to each other. For example, DNA for a presequence or secretory leader is operably linked to a polypeptide if it is expressed as a preprotein or participates in directing the polypeptide to the cell membrane or in secretion of the polypeptide. A promoter is operably linked to a coding sequence if it controls the transcription of the polypeptide; a ribosome binding site is operably linked to a coding sequence if it is positioned to permit translation. Usually, operably linked means contiguous and in reading frame, however, certain genetic elements such as repressor genes are not contiguously linked but still bind to operator sequences that in turn control expression.

Suitable host cells include prokaryotes, lower eukaryotes, and higher eukaryotes. Prokaryotes include both gram negative and gram positive organisms, e.g., *E. coli* and *B. subtilis*. Lower eukaryotes include yeasts, e.g., *S. cerevisiae* and *Pichia*, and species of the genus *Dictyostelium*. Higher eukaryotes include established tissue culture cell lines from animal cells, both of non-mammalian origin, e.g., insect cells,

and birds, and of mammalian origin, e.g., human, primates, and rodents.

Prokaryotic host-vector systems include a wide variety of vectors for many different species. As used herein, *E. coli* and its vectors will be used generically to include equivalent vectors used in other prokaryotes. A representative vector for amplifying DNA is pBR322 or many of its derivatives. Vectors that can be used to express the regulatory factor or its fragments include, but are not limited to, such vectors as those containing the lac promoter (pUC-series); trp promoter (pBR322-trp); Ipp promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as ptac (pDR540). See Brosius *et al.*, "Expression Vectors Employing Lambda-, trp-, lac-, and Ipp-derived Promoters", in *Vectors: A Survey of Molecular Cloning Vectors and Their Uses*, (eds. Rodriguez and Denhardt), 1988, Buttersworth, Boston, Chapter 10, pp. 205-236.

Lower eukaryotes, e.g., yeasts and *Dictyostelium*, may be transformed with regulatory factor sequence containing vectors. For purposes of this invention, the most common lower eukaryotic host is the baker's yeast, *Saccharomyces cerevisiae*. It will be used to generically represent lower eukaryotes although a number of other strains and species are also available. Yeast vectors typically consist of a replication origin (unless of the integrating type), a selection gene, a promoter, DNA encoding the regulatory factor or its fragments, and sequences for translation termination, polyadenylation, and transcription termination. Suitable expression vectors for yeast include such constitutive promoters as 3-phosphoglycerate kinase and various other glycolytic enzyme gene promoters or such inducible promoters as the alcohol dehydrogenase 2 promoter or metallothionine promoter. Suitable vectors include derivatives of the following types: self-replicating low copy number (such as the YRp-series), self-replicating high copy number (such as the

YEp-series); integrating types (such as the YIp-series), or mini-chromosomes (such as the YCp-series).

Higher eukaryotic tissue culture cells are the preferred host cells for expression of the functionally active regulatory factor protein. In principle, any higher eukaryotic tissue culture cell line is workable, e.g., insect baculovirus expression systems, whether from an invertebrate or vertebrate source. However, mammalian cells are preferred. Transformation or transfection and propagation of such cells has become a routine procedure. Examples of useful cell lines include HeLa cells, Chinese hamster ovary (CHO) cell lines, baby rat kidney (BRK) cell lines, insect cell lines, bird cell lines, and monkey (COS) cell lines.

Expression vectors for such cell lines usually include an origin of replication, a promoter, a translation initiation site, RNA splice sites (if genomic DNA is used), a polyadenylation site, and a transcription termination site. These vectors also usually contain a selection gene or amplification gene. Suitable expression vectors may be plasmids, viruses, or retroviruses carrying promoters derived, e.g., from such sources as from adenovirus, SV40, parvoviruses, vaccinia virus, or cytomegalovirus. Representative examples of suitable expression vectors include pCDNA1; pCD [Okayama *et al., Mol. Cell Biol. 5*:1136 (1985)]; pMC1neo PolyA [Thomas *et al., Cell 51*:503 (1987)]; and a baculovirus vector such as pAC 373 or pAC 610.

It will often be desired to express a regulatory factor polypeptide in a system which provides a specific or defined glycosylation pattern. In this case, the usual pattern will be that provided naturally by the expression system. However, the pattern will be modifiable by exposing the polypeptide, e.g., an unglycosylated form, to appropriate glycosylating proteins introduced into a heterologous expression system. For example, the regulatory factor gene may be co-transformed with one or more genes encoding

mammalian or other glycosylating enzymes. Using this approach, certain mammalian glycosylation patterns will be achievable in prokaryote or other cells.

Variants: Agonists and Antagonists

The isolated regulatory factor DNA can be readily modified by nucleotide substitutions, nucleotide deletions, nucleotide insertions, and inversions of nucleotide stretches. These modifications result in novel DNA sequences which encode these factors, their derivatives, or proteins having regulatory factor activity. These modified sequences can be used to produce mutant factors or to enhance the expression of the proteins. Enhanced expression may involve gene amplification, increased transcription, increased translation, and other mechanisms. Such mutant regulatory factor derivatives include predetermined or site-specific mutations of the respective regulatory factor or its fragments. A mutant regulatory factor refers to a polypeptide otherwise falling within the homology definition of the respective factor as set forth above, but having an amino acid sequence which differs from that of the factor as found in nature, whether by way of deletion, substitution, or insertion. In particular, "site specific mutant" is a factor with homology to a sequence defined by SEQ ID NOs 1-9 or 17, and as sharing various biological activities with those proteins.

Although site specific mutation sites are predetermined, mutants need not be site specific. Regulatory factor mutagenesis can be conducted by making amino acid insertions or deletions. Substitutions, deletions, insertions, or any combinations may be generated to arrive at a final construct. Insertions include amino- or carboxy- terminal fusions. Random mutagenesis can be conducted at a target codon and the expressed regulatory factor mutants can then be screened for the desired activity. Methods for making substitution mutations at predetermined sites in DNA having a

known sequence are well known in the art, e.g., by M13 primer mutagenesis. See also Sambrook *et al.* (1989) and Ausubel *et al.* (1987 and Supplements). PCR techniques, with or without additional synthetic oligonucleotide techniques will also be applicable. See, e.g., Innis et al. (eds), *PCR Protocols: A Guide to Methods and Applications*, 1990, Academic Press, NY; and Gait, *Oligonucleotide Synthesis: A Practical Approach,* 1984, IRL Press, Oxford.

The mutations in the DNA normally should not place coding sequences out of reading frames and preferably will not create complementary regions that could hybridize to produce secondary mRNA structure such as loops or hairpins.

The present invention also provides recombinant proteins, e.g., heterologous fusion proteins using segments from these regulatory factors. A heterologous fusion protein is a fusion of proteins or segments which are naturally not normally fused in the same manner. Thus, the fusion product of an immunoglobulin with a regulatory factor polypeptide is a continuous protein molecule having sequences fused in a typical peptide linkage, typically made as a single translation product and exhibiting properties derived from each source peptide. A similar concept applies to heterologous nucleic acid sequences.

In addition, new constructs may be made from combining similar functional domains from other proteins. For example, receptor-binding or other segments may be "swapped" between different new fusion polypeptides or fragments. See, e.g., Cunningham *et al., Science 243*:1330 (1989); and O'Dowd *et al., J. Biol. Chem. 263*:15985 (1988). Thus, new chimeric polypeptides exhibiting new combinations of specificities will result from the functional linkage of receptor-binding specificities from different factors. For example, the receptor binding domains from other related factors may be added or substituted for other receptor

binding domains of these factors. The resulting protein will often have hybrid function or properties.

The phosphoramidite method described by Beaucage and Carruthers, *Tetra. Letts. 22*:1859 (1981), will produce suitable synthetic DNA fragments. A double stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Antibodies

Antibodies can be raised to the modulator proteins described herein, and fragments thereof, both in their naturally occurring forms and in their recombinant forms. Additionally, antibodies can be raised to them in either their active forms or in their inactive forms, the difference being that antibodies to the active factor are more likely to recognize epitopes which are only present in the active conformation. Anti-idiotypic antibodies are also contemplated, and may be particularly important as agonists or antagonists.

Antibodies, including binding fragments and single chain versions, against predetermined fragments of the factors can be raised by immunization of animals with conjugates of the fragments with immunogenic proteins. Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies can be screened for binding to normal or defective factor, or screened for agonistic or antagonistic activity.

These monoclonal antibodies will usually bind with at least a K_D of about 1 mM, more usually at least about 300 μM, typically at least about 10 μM, more typically at least about 30 μM, preferably at least about 10 μM, and more preferably at least about 3 μM or better. Although the foregoing

addresses the specifically described embodiments, similar antibodies will be raised against other related factors and fragments thereof.

The antibodies, including antigen binding fragments, of this invention can have significant diagnostic or therapeutic value. They can be potent antagonists that bind to the factor and inhibit binding to a receptor or inhibit the ability of the factor to elicit a biological response. They also can be useful as non-neutralizing antibodies and can be coupled to toxins or radionuclides so that when the antibody binds to the factor, e.g., if expressed as a membrane bound protein, the cell itself is killed. Further, these antibodies can be conjugated to drugs or other therapeutic agents, either directly or indirectly by means of a linker.

The antibodies of this invention can also be useful in diagnostic applications. As capture or non-neutralizing antibodies, they can bind to the factor without inhibiting ligand binding. As neutralizing antibodies, they can be useful in competitive binding assays. They will also be useful in detecting or quantifying their respective factors.

Factor fragments may be joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides to be used as immunogens. Either factor and its fragments may be fused or covalently linked to a variety of immunogens, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc. See *Microbiology*, 1969, Hoeber Medical Division, Harper and Row; Landsteiner, *Specificity of Serological Reactions*, 1962, Dover Publications, New York; and Williams et al., *Methods in Immunology and Immunochemistry*, Vol. 1, 1967, Academic Press, New York, for descriptions of methods of preparing polyclonal antisera. A typical method involves hyperimmunization of an animal with an antigen. The blood of the animal is then collected shortly after the repeated immunizations and the gamma globulin is isolated.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of techniques for preparing such monoclonal antibodies may be found in, e.g., Stites *et al.* (eds), *Basic and Clinical Immunology* (4th ed.), Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, *Antibodies: A Laboratory Manual,* 1988, CSH Press; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed), 1986, Academic Press, New York; and particularly in Kohler and Milstein, *Nature 256*:495 (1975), which discusses one method of generating monoclonal antibodies.

Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Other suitable techniques involve *in vitro* exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors. See, Huse *et al., Science 246*:1275 (1989); and Ward *et al., Nature 341*:544 (1989).

The polypeptides and antibodies of the present invention may be used with or without modification, including chimeric or humanized antibodies and binding fragments thereof, e.g., Fab, Fv, etc. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are

known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents, teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced, see Cabilly, U.S. Patent No. 4,816,567.

The antibodies of this invention can also be used for affinity chromatography in isolating each factor. Columns can be prepared where the antibodies are linked to a solid support, e.g., particles, such as agarose, SEPHADEX® or the like, where a cell lysate may be passed through the column, the column washed, followed by increasing concentrations of a mild denaturant, whereby the purified regulatory factor protein will be released.

The antibodies may also be used to screen expression libraries for particular expression products. Usually the antibodies used in such a procedure will be labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

Antibodies raised against each factor will also be used to raise anti-idiotypic antibodies. These will be useful in detecting or diagnosing various immunological conditions related to expression of the respective factors.

Uses

Diagnostic and Therapeutic

The present invention provides better understanding of the mechanisms of regulation of hematopoietic progenitor cell development. In particular, the discovery of a new stimulator and inhibitor indicate their potential roles in conditions which exhibit symptoms of abnormal patterns of hematopoietic cell

lineage populations. For example, conditions where particular cell types are overexpressed will be diagnosed for overproduction of the HR1 stimulator factor or underproduction of the HR2 inhibitory factor.

Where particular cell types are underexpressed, the converse will be checked. Typically, these conditions will be those where defined lineages are overproduced, e.g., leukemias, lymphomas, and gammapathies, both monoclonal or polyclonal. Alternatively, conditions where defined lineages are underproduced, e.g., anemias, will also be checked.

These deficiencies, if detected, may be treated therapeutically by supplementing the relevant factor. Administration may be either systemic or localized, as appropriate.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for in situ administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, e.g., in Gilman *et al.* (eds), *Goodman and Gilman's: The Pharmacological Bases of Therapeutics,* 8th Ed., 1990, Pergamon Press; and *Remington's Pharmaceutical Sciences,* 17th ed., 1990, Mack Publishing Co., Easton, Penn.

Methods for administration are discussed therein and below, e.g., for oral, intravenous, intraperitoneal, or intramuscular administration, transdermal diffusion, and others. Pharmaceutically acceptable carriers will include water, saline, buffers, and other compounds described, e.g., in the *Merck Index,* Merck & Co., Rahway, New Jersey. Low

dosages of these reagents would be preferred, and likely to be effective. Thus, dosage ranges would ordinarily be expected to be in amounts lower than 1 mM concentrations, typically less than about 10 μM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an appropriate carrier. Slow release formulations, or slow release apparatus will often be utilized for continuous administration.

The regulatory factor, fragments thereof, and antibodies to the factor or its fragments, antagonists, and agonists, may be administered directly to the host to be treated or, depending on the size of the compounds, it may be desirable to conjugate them to carrier proteins such as ovalbumin or serum albumin prior to their administration. Therapeutic formulations may be administered in any conventional dosage formulation. While it is possible for the active ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation.

Formulations comprise at least one active ingredient, as defined above, together with one or more acceptable carriers thereof. Each carrier must be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration.

The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. See, e.g., Gilman *et al.* (eds), *supra*; and *Remington's Pharmaceutical Sciences, supra.*

Antibodies

Antibodies, including antigen binding fragments, specific for the regulatory factor or peptide fragments thereof are useful in diagnostic applications to detect the presence of

elevated levels of the factor and/or its fragments. Such diagnostic assays can employ lysates, live cells, fixed cells, immunofluorescence, cell cultures, body fluids, and further can involve the detection of antigens related to the regulatory factor in serum, or the like. Diagnostic assays may be homogeneous (without a separation step between free reagent and reagent-target complex) or heterogeneous (with a separation step). Various commercial assays exist, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), enzyme-multiplied immunoassay technique (EMIT), substrate-labeled fluorescent immunoassay (SLFIA) and the like. For example, unlabeled antibodies can be employed by using a second antibody which is labeled and which recognizes the antibody to the regulatory factor or to a particular fragment thereof. These assays have also been extensively discussed in the literature. See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, 1988, Cold Spring Harbor Press, CSH, NY.

Anti-idiotypic antibodies may have similar use to diagnose presence of antibodies against a factor, as such may be diagnostic of various abnormal states. For example, overproduction of a regulatory factor may result in production of various immunological reactions which may be diagnostic of abnormal factor expression, particularly in hematopoietic cell proliferative abnormalities. Particularly, leukemias, lymphomas, and monoclonal gammapathies may result from abnormal expression of regulatory factors. The conditions may arise from overproduction of stimulating factors or underproduction of inhibitory factors. Likewise, deficiencies of particular cell lineages may be due to the converse imbalance in expression of regulatory factors.

Kits

This invention also contemplates use of these factors, fragments thereof, peptides, and their fusion products in a variety of diagnostic kits and methods for detecting the presence of the regulators. Typically the kit will have a compartment containing either a defined HR peptide or gene segment, e.g., as a positive control, or a reagent which recognizes one or the other.

A kit for determining the binding affinity of a test compound to the desired HR would typically comprise a test compound; a labeled compound, e.g., an antibody having known binding affinity for the regulatory factor; a source of factor (naturally occurring or recombinant); and a means for separating bound from free labeled compound, such as a solid phase for immobilizing the regulatory factor. Agonists or antagonists may be derived from anti-idiotypic antibodies against the regulatory factors. The availability of recombinant factor polypeptides also provide well defined standards for calibrating such assays.

A preferred kit for determining the concentration of, e.g., HR1, in a sample would typically comprise a labeled compound, e.g., antibody, having known binding affinity for HR1, a source of HR1 as a positive control (naturally occurring or recombinant), and a means for separating the bound from free labeled compound, for example a solid phase for immobilizing the HR1. Compartments containing reagents, and instructions, will normally be provided.

Frequently, the reagents for diagnostic assays are supplied in kits, so as to optimize the sensitivity of the assay. For the subject invention, depending upon the nature of the assay, the protocol, and the label, either labeled or unlabeled antibody, or labeled factor is provided. This is usually in conjunction with other additives, such as buffers, stabilizers, materials necessary for signal production such as substrates for enzymes, and the like. Preferably, the kit will also contain

instructions for proper use and disposal of the contents after use. Typically the kit has compartments for each useful reagent. Desirably, the reagents are provided as a dry lyophilized powder, where the reagents may be reconstituted in an aqueous medium having appropriate concentrations for performing the assay.

Any of the diagnostic assays may be used without modification or may be modified in a variety of ways. For example, labeling may be achieved by covalently or non-covalently joining a moiety which directly or indirectly provides a detectable signal. In any of these assays, the test compound, regulatory factor, or antibodies thereto can be labeled either directly or indirectly. Possibilities for direct labeling include label groups: radiolabels such as ^{125}I, enzymes (U.S. Pat. No. 3,645,090) such as peroxidase and alkaline phosphatase, and fluorescent labels (U.S. Pat. No. 3,940,475) capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization. Possibilities for indirect labeling include biotinylation of one constituent followed by binding to avidin coupled to one of the above label groups.

There are also numerous methods for separating the bound from the free ligand, or alternatively the bound from the free test compound. The regulatory factor can be immobilized on various matrices followed by washing. Suitable matrices include plastic such as an ELISA plate, filters, and beads. Methods of immobilizing the regulatory factor to a matrix include, without limitation, direct adhesion to plastic, use of a capture antibody, chemical coupling, and biotin-avidin. The last step in this approach involves the precipitation of binding component-target complex by any of several methods including those utilizing, e.g., an organic solvent such as polyethylene glycol or a salt such as ammonium sulfate. Other suitable separation techniques include, without limitation, the fluorescein antibody

magnetizable particle method described by Rattle *et al.*, *Clin. Chem. 30*:1457 (1984), and the double antibody magnetic particle separation described in U.S. Pat. No. 4,659,678.

Methods for linking proteins or their fragments to various labels have been extensively reported in the literature and do not require detailed discussion here. Many of the techniques involve the use of activated carboxyl groups either through the use of carbodiimide or active esters to form peptide bonds, the formation of thioethers by reaction of a mercapto group with an activated halogen such as chloroacetyl, or an activated olefin such as maleimide, for linkage, or the like. Fusion proteins will also find use in these applications.

Another diagnostic aspect of this invention involves use of oligonucleotide or polynucleotide sequences taken from the sequence of a regulatory factor. These sequences can be used as probes for detecting levels of the message in patients suspected of having abnormal expression of hematopoietic regulatory factors, e.g., indications described above. The preparation of both RNA and DNA nucleotide sequences, the labeling of the sequences, and the preferred size of the sequences has received ample description and discussion in the literature. Normally an oligonucleotide probe should have at least about 14 nucleotides, usually at least about 18 nucleotides, and the polynucleotide probes may be up to several kilobases. Various labels may be employed, most commonly radionuclides, particularly ^{32}P. However, other techniques may also be employed, such as using biotin modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed which can recognize specific duplexes, including DNA duplexes, RNA duplexes, DNA-RNA hybrid duplexes, or DNA-protein

duplexes. The antibodies in turn may be labeled and the assay carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected. The use of probes to the novel anti-sense RNA may be carried out in any conventional techniques such as nucleic acid hybridization, plus and minus screening, recombinational probing, hybrid released translation (HRT), and hybrid arrested translation (HART). This also includes amplification techniques such as polymerase chain reaction (PCR).

Diagnostic kits which also test for the qualitative or quantitative presence of other markers are also contemplated. Diagnosis or prognosis may depend on the combination of multiple indications used as markers. Thus, kits may test for combinations of markers. See, e.g., Viallet *et al., Progress in Growth Factor Res. 1*:89 (1989).

Similar reagents are made available for application of these concepts to other variants of these regulatory factors.

EXAMPLES

The present invention can be illustrated by the following, non-limiting Example. Unless otherwise specified, percentages given below for solids in solid mixtures, liquids in liquids, and solids in liquids are on a wt/wt, vol/vol and wt/vol basis, respectively. Sterile conditions were maintained during cell culture.

Reagents and Methods

Cell Line Supernatants

Supernatants from hematopoietic and non-hematopoietic cells were tested for their ability to stimulate erythropoiesis *in vitro*. B cell: CH12 [sublines LX, LX4867, 4550 from A. O'Garra, DNAX; see Haughton *et al., Immunol. Rev. 93*:35

(1986)]: Colon: Colo P3 ; erythroid: K562, MEC 3.6 ; keratinocyte: Pamm 134; macrophage: 1G18LA (thymic-derived from A. Zlotnik, DNAX); P388D1 (ATCC CCL46); stromal: 30R (bone marrow-derived from D. Rennick, DNAX): Th1 T cells: [see Mosmann *et al., Proc. Nat'l Acad Sci.* USA *136*:2348 (1986) and Fiorentino *et al., J. Exptl. Med. 170*:2081 (1989) for the following: D1.1, H66.61, LB21, M2640-20.037, MD15-5.1, MLA51]; and from Th2 T cells: CDC-25/8 (Tony *et al., J. Exptl. Med. 161*:223 (1985); D9 [Nabel *et al., Proc. Natl. Acad. Sci.* USA *78*:1157 (1981)]; D10.G4.1 [Kaye *et al., J. Exptl. Med. 158*:836 (1983)]; MB2-1, [Mosmann *et al. Proc. Natl. Acad. Sci.* USA *136*:2348 (1986)].

Growth and Preparation of D10 Supernatants

T cell growth medium consisted of RPMI 1640 (JRH Scientific, Belmont, CA) or a supplemented medium, AIMV (Gibco Laboratories, Grand Island, NY), containing 5-10% fetal calf serum (JRH Scientific), 0.05 mM beta-mercaptoethanol (Sigma Chemical Company, St. Louis, MO), 20 mM Hepes (Gibco Laboratories, Grand Island, NY), 10,000 U/ml of penicillin-streptomycin (Gibco Laboratories, Grand Island, NY), and 500 U/ml of recombinant IL-2 (G. Zurawski, DNAX).

To maximize cytokine production, D10 cells were exposed to antigen 1-3 weeks prior to induction [Mosmann *et al., Proc. Natl. Acad. Sci.* USA *136*:2348 (1986)]. D10 cells (5×10^5 cells/ml) were exposed to 100 µg/ml of conalbumin (Sigma Chemical Company) and irradiated (3-4 week old) BALB/c spleens (Simonsen Laboratories, Gilroy, CA) at a concentration of 5×10^6 cells/ml were aliquoted into individual wells in T cell growth medium. One to two weeks later, D10 cells were harvested and induced by incubating D10 cells at a concentration of 10^6 cells/ml in T cell growth medium without fetal calf serum and 5 µg/ml concanavalin A (Sigma Chemical Company, St. Louis, MO). Supernatants were harvested after 24 hours.

Cytokine ELISAs

Two-site sandwich ELISAs were used to measure murine cytokines in supernatants included: IL-3 [Abrams *et al., J. Immunol. 140*:131 (1988)], IL-4 [Ohara *et al., Nature 315*:333 (1985)], IL-5 [Schumacher *et al., J. Immunol. 141*:1576 (1988)], IL-6 [Starnes *et al., J. Immunol. 145*:4185 (1990)], IL-10 [Mosmann *et al., J. Immunol. 145*:2938 (1990)], gamma-interferon [Cherwinski *et al., J. Exptl. Med. 166*:1229 (1987)], and GM-CSF (J. Abrams, DNAX, Palo Alto, CA).

Column Chromatography

Serum-free ConA-induced D10 cell supernatants (6-9 liter batches) were concentrated about ten-fold using YM-10 membranes (Amicon Corporation, Danvers, MA), passed through a 5 ml mannose-conjugated agarose column (E-Y Laboratories, San Mateo, CA), and further concentrated another three-to-five-fold. This provided a total concentration of about 30-50-fold. The concentrated material was fractionated sequentially by HPLC on a hydroxylapatite-based support (Bio-Gel HPHT, 7.5 x 75 mm, Bio-Rad Laboratories, Richmond, CA: or TSK-gel HA1000, 21.5 x 150 mm, Novex, San Diego, CA), samples were applied to the column and washed in 10 mM NaPi pH 7.4, 0.12 mM $CaCl_2$, and 0.01% Tween-20; and eluted in a gradient buffer reaching 350 mM NaPi, pH 7.4, 3.5 µM $CaCl_2$, and 0.01% Tween-20; a hydrophobic interaction-based chromatography support (Bio-Gel TSK phenyl 5-PW, 7.5 mm x 75 mm, Bio-Rad Laboratories, Hercules, CA). Samples were applied to the column and washed in 1 M Na_2SO_4, 0.1 M NaPi, pH 7.0; and eluted in a gradient reaching 0.05 M NaPi, pH 7.0, and 50% HO-$(CH_2)_2$-OH (ethylene glycol; see Uyttenhove *et al., Proc. Natl. Acad. Sci.* USA *85*:6934 (1988); a gel filtration column (TSK-G2000SW, 7.5 x 600 mm, LKB Instruments, Gaithersburg, MD) run in 2X CBSS (CBSS = complete balanced salt solution; 1X is 0.137 M

NaCl, 5 mM KCl, 1.3 mM Na_2HPO_4, 0.3 mM KH_2PO_4, and 5.6 mM glucose; see Bond *et al., J. Immunol. 139*:3691 (1987) and 0.01% Tween-20; and either on a reverse phase column (Aquapore BU-300, Brownlee Division, Applied Biosystems, Foster City, CA; or Poros R/H, PerSeptive, Cambridge, MA), samples were applied to the column in 0.1% trifluoroacetic acid (TFA), 10% CH_3CN (acetonitrile) and eluted in a gradient reaching 0.1% TFA, 70% CH_3CN; or an anion exchange column (Poros Q/H, 2.1 x 30 mm, PerSeptive Biosystems, Cambridge, MA), samples were applied to the column in 20 mM $(HOCH_2CH_2)_3N^+Cl^-$ (triethanolamine), pH 7.5, 0.01% Tween-20, and eluted in a gradient of the same buffer with NaCl up to 1 M. Molecular weights in size exclusion chromatography were determined by comparison to elution times of a mixture of standards (United States Biochemical Corporation, Cleveland, OH).

Anti-cytokine Affinity Columns

Five affinity columns were prepared by coupling anti-murine cytokine monoclonal antibodies at a concentration of between 5-20 mg/ml to a support matrix (Affi-Gel 10; BioRad Laboratories, Richmond, CA) in a total volume of 5 ml. Antibodies included: anti-IL-3, 8F8.11 [Abrams *et al., J. Immunol. 140*:131 (1988)]; anti-IL-4, 11B11 [Ohara *et al., Nature 315*:333 (1985)]; anti-IL-5, TRFK5 [Schumacher *et al., J. Immunol. 141*:1576 (1988)]; anti-IL-6, MP5-20F3 [Starnes *et al., J. Immunol. 145*:4185 (1990)]; and anti-GM-CSF, 22E9.11 (J. Abrams, DNAX, Palo Alto, CA). ELISA's confirmed cytokine depletion at greater than 95%.

Colony Forming Units-erythroid (CFU-E) Liquid Assay

A rapid, liquid assay was developed to detect stimulators or inhibitors of CFU-E differentiation. Fetal livers from BALB/c mice (Simonsen Laboratories, Gilroy, CA) were harvested at day of 13 gestation. The dissociated cells were

plated in 96 well flat bottom plates (Falcon) at a concentration of 6.5 x 10^5 cells/ml in IMDM (Gibco Laboratories, Grand Island, NY) containing 100 µg/ml transferrin (Sigma Chemical Company, St. Louis, MO), 25 µg/ml purified bovine insulin (Sigma Chemical Co.), penicillin-streptomycin (Gibco Laboratories, Grand Island, NY) at 100 U/ml and 100 µg/ml, respectively, and 0.05 mM beta-mercaptoethanol (Sigma Chemical Company, St. Louis, MO). Test samples were routinely diluted over a 1,000-fold range and cultured in a humidified incubator at 37 °C in 5% CO_2 for either two or three days. In longer experiments 500 mU/ml of human erythropoietin (Amgen Diagnostics, Thousand Oaks, CA, or R & D Systems, Inc., Minneapolis, MN) was added after 24 hours. Viability was measured using a spectrophotometric readout.

Specifically, a tetrazolium salt MTT [Mosmann, *J. Immunol. Methods 65*:95 (1983)] or XTT [Roehm *et al.*, *J. Immunol. Methods 142*:257 (1991)] is cleaved by dehydrogenase enzymes in metabolically active cells. The resulting cleavage product is highly colored and can be distinguished spectrophotometrically from the starting compound. A benzidine-like stain, 2,7-diaminofluorene (Sigma Chemical Co., St. Louis, MO) confirmed that a majority of cells in the cultures were erythroid. See Roehm *et al.*, *J. Immunol. Methods 142*:257 (1991). An arbitrary determination of activity was made in each experiment by establishing the maximum stimulation and assigning the value of 1 U/ml to the dilution corresponding to half maximum. Actions of known cytokines were determined in the assay.

This assay provides substantial advantages over other assays in being rapid and easily read. As a colorimetric assay, it was subject to high volume microtiter plate spectrophotometric analysis. Use of microtiter plates made the assays compatible with existing equipment, and allowed screening large numbers of samples. Finally, the sensitivity

and reproducibility allowed detection over a 1000-fold range of activity.

Elution of Biologically Active Material from SDS-PAGE

Protein was eluted from SDS gels after appropriate migration position was determined by a parallel track of protein which was silver stained.

Amino Acid Analysis

Purified protein was digested in a 200 µl volume of 100 mM Tris-HCl, pH 7.5, with 0.1% Tween-20 (BioRad, Richmond, CA) and 0.5 µg of trypsin (sequencing grade, Boehringer-Mannheim, Indianapolis, IN). Enzyme was added in two equal aliquots, at the outset and at 8 hours. Digestion was carried out at 37 °C for 16 hours. Peptides were separated on a Brownlee RP-300 2.1 x 30 mm butyl narrow bore column, using an ABI 140B syringe driven pump with microbore tubing (Applied Biosystems, Foster City, CA).

Sequence analysis was performed on an ABI gas phase sequencer (Model 477A, Applied Biosystems, Foster City, CA) with an online PTH amino acid analyzer (Model 120A, Applied Biosystems, Foster City, CA.

Observation of Novel Modulator Activities

The newly developed liquid CFU-E assay was used to screen supernatants from a broad range of cell types, including hematopoietic-derived B and T cell, macrophage, stromal, and erythroleukemic lines and non-hematopoietic colon and keratinocyte cell lines. Supernatants from antigen-stimulated T cells were the most potent. Other stimulators of *in vitro* erythropoiesis (IL-3, IL-4, and GM-CSF) are secreted in high abundance by T cells [see Mosmann *et al., Proc. Natl. Acad. Sci.* USA *136*:2348 (1986); Fiorentino *et al., J. Exptl Med.* *170*:2081 (1989)]. Supernatants from the mouse T cell line,

D10.G4.1 (D10) [Kaye *et al., J. Exptl. Med. 158*:836 (1983)] were selected for further characterization.

A stimulatory activity was observed, and the activity was fractionated by various chromatography methods. Also among the various activities was observed a separable inhibitory activity.

Purification of HR1 Proteins

The HR1 class of proteins were identified as factors which posses a biological activity of stimulating hematopoietic, e.g., erythroid development. These proteins are characterized herein as a 38 kD protein, as described above.

Pooled batches of D10 conditioned medium were concentrated and passed through a mannose lectin column to remove ConA. The resulting material was subjected to five anti-cytokine affinity columns, as described above, to remove known cytokines (IL-3, IL-4, IL-5, IL-6, GM-CSF).

A CFU-E stimulating activity was followed though a series of steps of biochemical columns. A hydroxylapatite-based column concentrated the biological activity into a uniform region, and a hydrophobic interaction-based column distributed the activities over a broad range. The predominant CFU-E activity detected in the hydrophobic interaction column was observed in three separate batches of D10.

Pooled fractions in this region were passed through a gel filtration column. The uniform peak of CFU-E activities in gel filtration columns were followed either separately or pooled together on subsequent columns. In either case, the CFU-E activity was associated with a characteristic mixture of proteins in the flow through of an anion exchange column. The CFU-E activity in this region was unstable in acetonitrile-trifluoroacetic acid, preventing purification of the biologically active component by reverse phase chromatography.

As an alternative, attempts were made to isolate the CFU-E activity by elution from SDS-PAGE (material was run on an adjacent lane and the gel silver stained to identify proteins). The SDS-protein complex apparently was toxic to the erythroid progenitors, preventing detection of biological activity in the assay.

Throughout the purification process, other murine hematopoietic target cells were used, including enriched bone-marrow derived progenitors [Sca-/lin- phenotype, Heimfeld *et al., Proc. Natl Acad. Sci.* USA *88*:9902 (1991), a mast cell progenitor line, MC9, Nabel *et al., Proc. Natl. Acad. Sci.* USA *78*:1157 (1981), and a myeloid progenitor line, DA1, Weinstein *et al., Proc. Natl. Acad. Sci.* USA *83*:5010 (1986)]. This served not only to help identify known cytokines that were not removed by antibody affinity columns but also to screen for novel cytokines. A similar pattern of activity for these target cells was seen following anion exchange column chromatography.

Samples from the gel slice elution experiments were assayed on these target cells. SDS apparently was toxic on the mast cell and myeloid cell lines, but the enriched bone marrow-derived progenitors identified an activity that was associated with a 38 Kd protein.

In a previous batch of D10 conditioned medium, the 38 kD protein was purified to homogeneity by passing the flow through of an anion exchange column through a reverse phase column. The 38 kD protein from the two batches appeared identical when compared by SDS-PAGE. Adequate quantities of biologically inactive material was available for further analysis. The protein was digested with trypsin and peptide fragments were purified by narrow bore reverse phase chromatography. The amino acid sequences of individual peptides (SEQ ID NOs 1-9) were obtained by standard methods. Recombinant *E. coli*-derived gCap39 was

tested for effect on erythroid progenitor cells *in vitro*, and a stimulatory effect was observed.

Characterization of HR1 Protein

Sequence data from more than a third of the 38 kD protein was compared to sequences found in the Swissprot and PIR/NBR sequence databases and two other proteins showed near identity. One protein, Mbh1, was identified as a possible member of a complex of proteins associated with c-myc in DNA binding (Prendergast *et al., EMBO J.*). The other protein, gCap39, was identified by looking for more members of the actin-binding protein family, gelsolin. See Stossel, *J. Biol. Chem. 264*:18261 (1989).

Purification of HR2 Protein

Similar techniques were applied to isolation of the inhibitory activity. The activity was fractionated using the same techniques, although the exact locations of elution differed from those of the HR1 class of proteins.

Characterization of HR2 Protein

Amino acid sequence data from the isolated activity provided minimal sequence data. The N-terminus of the regulatory factor is blocked, but CNBr treatment yielded a fragment having the amino terminal sequence LFGKIVNR. In searching through protein sequence data bases, matches for this short sequence identified various members of the int/wnt class of proteins. Using a PCR-based strategy, various related cDNA species have been identified from cDNA libraries from mouse T cells. The proteins are of similar size and share 50% to 85% amino acid identity with 83 absolutely conserved

amino acids. However, the protein has 21 cysteine residues. See Gavin *et al., Genes & Development 4*:2319 (1990).

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will become apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Schering Corporation
(B) STREET: One Giralda Farms
(C) CITY: Madison
(D) STATE: New Jersey
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 07940-1000
(G) TELEPHONE: 201-822-7375
(H) TELEFAX: 201-822-7039
(I) TELEX: 219165

(ii) TITLE OF INVENTION: Modulators of Hematopoietic Progenitor Cells

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 6.0.5
(D) SOFTWARE: Microsoft Word 5.1a

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/976,789
(B) FILING DATE: 16-NOV-1992

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Glu Val Gln Gly Asn Glu Thr Asp Leu Phe Met Ser Tyr Phe Pro
1               5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Glu Gly Gly Val Glu Ser Ala Phe His
1               5
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Asp Leu Ala Leu Ala Ile Xaa
1               5
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Ala Gln Val Glu Ile Ile Thr Xaa Gly Ile Xaa Pro Ala
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Glu Gly Asn Pro Glu Glu Asp Ile Thr Ala Asp Gln Thr Xaa Asn Ala
1               5                   10                  15

Gln Ala Ala Ala Leu Tyr
            20
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Val Asn Ile Ala Thr Gly Gln Met Asn Leu Thr
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Val Ala Asp Ser Ser Pro Phe Ala Xaa Glu Leu Leu Ile Pro Asp Asp
1               5                   10                  15
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Gln Ala Ala Leu Gln Val Ala Asp Gly Phe Ile Ser
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Tyr Ser Pro Asn Thr Gln Val Glu Ile Leu Pro Gln Gly Gln Leu
1               5                   10                  15
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 352 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Tyr Thr Pro Ile Pro Gln Ser Gly Ser Pro Phe Pro Ala Ser Val
1               5                  10                  15

Gln Asp Pro Gly Leu His Ile Trp Arg Val Glu Lys Leu Lys Pro Val
            20                  25                  30

Pro Ile Ala Arg Glu Ser His Gly Ile Phe Phe Ser Gly Asp Ser Tyr
        35                  40                  45

Leu Val Leu His Asn Gly Pro Glu Glu Ala Ser His Leu His Leu Trp
    50                  55                  60

Ile Gly Gln Gln Ser Ser Arg Asp Glu Gln Gly Ala Cys Ala Val Leu
65                  70                  75                  80

Ala Val His Leu Asn Thr Leu Leu Gly Glu Arg Pro Val Gln His Arg
                85                  90                  95

Glu Val Gln Gly Asn Glu Ser Asp Leu Phe Met Ser Tyr Phe Pro Arg
            100                 105                 110

Gly Leu Lys Tyr Tyr Arg Glu Gly Gly Val Glu Ser Ala Phe His Lys
        115                 120                 125

Thr Thr Ser Gly Ala Arg Gly Ala Ala Ile Arg Lys Leu Tyr Gln Val
    130                 135                 140

Lys Gly Lys Lys Asn Ile Arg Ala Thr Glu Arg Pro Leu Ser Trp Asp
145                 150                 155                 160

Ser Phe Asn Thr Gly Asp Cys Phe Ile Leu Asp Leu Gly Gln Asn Ile
                165                 170                 175

Phe Ala Trp Cys Gly Gly Lys Ser Asn Ile Leu Glu Arg Asn Lys Ala
            180                 185                 190

Arg Asp Leu Ala Leu Ala Ile Arg Asp Ser Glu Arg Gln Gly Lys Ala
        195                 200                 205

Gln Val Glu Ile Ile Thr Asp Gly Glu Glu Pro Ala Glu Met Ile Gln
    210                 215                 220

Val Leu Gly Pro Lys Pro Ala Leu Lys Glu Gly Asn Pro Glu Glu Asp
225                 230                 235                 240

Ile Thr Ala Asp Gln Thr Arg Pro Asn Ala Gln Ala Ala Ala Leu Tyr
                245                 250                 255
```

```
Lys Val Ser Asp Ala Thr Gly Gln Met Asn Leu Thr Lys Val Ala Asp
            260                 265                 270

Ser Ser Pro Phe Ala Ser Glu Leu Leu Ile Pro Asp Asp Cys Phe Val
        275                 280                 285

Leu Asp Asn Gly Leu Cys Ala Gln Ile Tyr Ile Trp Lys Gly Arg Lys
    290                 295                 300

Ala Asn Glu Lys Glu Arg Gln Ala Ala Leu Gln Val Ala Asp Gly Phe
305                 310                 315                 320

Ile Ser Arg Met Arg Tyr Ser Pro Asn Thr Gln Val Glu Ile Leu Pro
                325                 330                 335

Gln Gly Arg Glu Ser Pro Ile Phe Lys Gln Phe Phe Lys Asn Trp Lys
            340                 345                 350
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 353 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Met Tyr Thr Pro Ile Pro Gln Ser Gly Ser Pro Phe Pro Ala Ser Val
1               5                   10                  15

Gln Asp Pro Gly Leu His Ile Trp Arg Val Glu Lys Leu Lys Pro Val
            20                  25                  30

Pro Ile Ala Arg Glu Ser His Gly Ile Phe Phe Ser Gly Asp Ser Tyr
        35                  40                  45

Leu Val Leu His Asn Gly Pro Glu Glu Ala Ser His Leu His Leu Trp
    50                  55                  60

Ile Gly Gln Gln Ser Ser Arg Asp Glu Gln Gly Ala Cys Ala Val Leu
65                  70                  75                  80

Ala Val His Leu Asn Thr Leu Leu Gly Glu Arg Pro Val Gln His Arg
                85                  90                  95

Glu Val Gln Gly Asn Glu Ser Asp Leu Phe Met Ser Tyr Phe Pro Arg
            100                 105                 110

Gly Leu Lys Tyr Tyr Arg Glu Gly Gly Val Glu Ser Ala Phe His Lys
        115                 120                 125
```

```
Thr Thr Ser Gly Ala Arg Gly Ala Ala Ile Arg Lys Leu Tyr Gln Val
    130                 135                 140

Lys Gly Lys Lys Asn Ile Arg Ala Thr Glu Arg Pro Leu Ser Trp Asp
145                 150                 155                 160

Ser Phe Asn Thr Gly Asp Cys Phe Ile Leu Asp Leu Gly Gln Asn Ile
                165                 170                 175

Phe Ala Trp Cys Gly Gly Lys Ser Asn Ile Leu Glu Arg Asn Lys Ala
            180                 185                 190

Arg Asp Leu Ala Leu Ala Ile Arg Asp Ser Glu Arg Gln Gly Lys Ala
        195                 200                 205

Gln Val Glu Ile Ile Thr Asp Gly Glu Glu Pro Ala Glu Met Ile Gln
    210                 215                 220

Val Leu Gly Pro Lys Pro Ala Leu Lys Glu Gly Asn Pro Glu Glu Asp
225                 230                 235                 240

Ile Thr Ala Asp Gln Thr Arg Pro Asn Ala Gln Ala Ala Ala Leu Tyr
                245                 250                 255

Lys Val Ser Asp Ala Thr Gly Gln Met Asn Leu Thr Lys Val Ala Asp
            260                 265                 270

Ser Ser Pro Phe Ala Ser Glu Leu Leu Ile Pro Asp Asp Cys Phe Val
        275                 280                 285

Leu Asp Asn Gly Leu Cys Ala Gln Ile Tyr Ile Trp Lys Gly Arg Lys
    290                 295                 300

Ala Asn Glu Lys Glu Arg Gln Ala Ala Leu Gln Val Ala Asp Gly Phe
305                 310                 315                 320

Ile Ser Arg Met Arg Tyr Ser Pro Asn Thr Gln Val Glu Ile Leu Pro
                325                 330                 335

Gln Gly Arg Glu Ser Pro Ile Phe Lys Gln Phe Phe Lys Asn Trp Lys
            340                 345                 350

Val
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Leu Phe Gly Lys Ile Val Asn Arg
1               5
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GATCGCGGCC GCCARGARTG YAARTGYCAT 30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTACCCGCGG RCARCACCAR TGRAA 25

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 216 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
CATGGCACAT CAGGCAGCTG CCAGTTCAAG ACATGCTGGA GGGCGGCCCC AGAGTTCCGG  60

GCAGTGGGGG CGGCGTTGAG GGAGCGGCTG GGCCGGGCCA TCTTCATTGA TACCCACAAC 120

CGCAATTCTG GAGCCTTCCA GCCCCGTCTG CGTCCCCGTC GCCTCTCAGG AGAGCTGGTC 180

TACTTTGAGA AGTCTCCTGA CTTCTGTGAG CGAGAC                           216
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2233 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
CTCGAGCAGA ACCACCCGTG AGTTAGGTCG AGCAGAGCCA AAGCCCCCGG TGCTTCGTCG  60

CGGGTTCGCT CGCTAGCTAT CTGGATCACT CCCTCCCTTT TACCCTCCCT TCCTCCCGGC 120

GGGCGGCCGC GGCGACGCCG GGGAAGCGGC AGAGAGGAGT GGCTGGGCGC TGGGAGAATG 180

CTGCTCCGCC GAGGGGGCTG AACCCGACAG TTTCCCCACG GTTTAAGCCC CAAGAGCCGG 240

GCCCGAGTGA CTCAACCGCG AGCCTTGTGG ATCCTGCACC TGAACCGCTG GAGGCTGACT 300

GACTCGCCCA CCGGAGCCTC CGGGCTTCGA C ATG CTG GAG GAG CCC CGG TCT    352
                                  Met Leu Glu Glu Pro Arg Ser
                                    1               5

CGG CCT CCG CCC TTA GGC CTC GCG GGT CTC CTG TTC TTG GCT TTG TTC   400
Arg Pro Pro Pro Leu Gly Leu Ala Gly Leu Leu Phe Leu Ala Leu Phe
        10                  15                  20

AGT CGG GCT CTA AGC AAT GAG ATT CTG GGC CTT AAA CTT CCC GGT GAG   448
Ser Arg Ala Leu Ser Asn Glu Ile Leu Gly Leu Lys Leu Pro Gly Glu
    25                  30                  35

CCG CCG CTG ACG GCC AAC ACC GTG TGC TTG ACC CTG TCC GGA CTG AGT   496
Pro Pro Leu Thr Ala Asn Thr Val Cys Leu Thr Leu Ser Gly Leu Ser
 40                  45                  50                  55

AAG CGA CAG CTG GGG CTG TGC CTG CGC AGC CCC GAC GTG ACG GCG TCG   544
Lys Arg Gln Leu Gly Leu Cys Leu Arg Ser Pro Asp Val Thr Ala Ser
                60                  65                  70
```

```
GCG CTC CAG GGG CTG CAC ATC GCC GTT CAC GAG TGT CAG CAC CAG CTG   592
Ala Leu Gln Gly Leu His Ile Ala Val His Glu Cys Gln His Gln Leu
             75                  80                  85

CGC GAC CAG CGC TGG AAC TGC TCG GCA CTG GAG GGC GGC GGC CGG CTG   640
Arg Asp Gln Arg Trp Asn Cys Ser Ala Leu Glu Gly Gly Gly Arg Leu
         90                  95                 100

CCG CAC CAC AGC GCC ATC CTC AAG CGC GGT TTC CGT GAG AGT GCT TTC   688
Pro His His Ser Ala Ile Leu Lys Arg Gly Phe Arg Glu Ser Ala Phe
    105                 110                 115

TCC TTC TCC ATG CTG GCT GCT GGG GTC ATG CAT GCT GTT GCC ACA GCC   736
Ser Phe Ser Met Leu Ala Ala Gly Val Met His Ala Val Ala Thr Ala
120                 125                 130                 135

TGC AGC CTG GGC AAG CTG GTG AGC TGC GGC TGC GGA TGG AAG GGT AGT   784
Cys Ser Leu Gly Lys Leu Val Ser Cys Gly Cys Gly Trp Lys Gly Ser
                140                 145                 150

GGT GAG CAA GAC CGG CTT AGA GCC AAG CTG CTG CAG CTT CAG GCA CTG   832
Gly Glu Gln Asp Arg Leu Arg Ala Lys Leu Leu Gln Leu Gln Ala Leu
            155                 160                 165

TCT CGG GGC AAG ACT TTC CCC ATC TCC CAG CCC AGC CCT GTT CCT GGC   880
Ser Arg Gly Lys Thr Phe Pro Ile Ser Gln Pro Ser Pro Val Pro Gly
        170                 175                 180

TCA GTC CCC AGC CCC GGC CCC CAG GAC ACG TGG GAA TGG GGT GGC TGT   928
Ser Val Pro Ser Pro Gly Pro Gln Asp Thr Trp Glu Trp Gly Gly Cys
    185                 190                 195

AAC CAC GAC ATG GAC TTC GGA GAG AAG TTC TCT CGG GAT TTC TTG GAT   976
Asn His Asp Met Asp Phe Gly Glu Lys Phe Ser Arg Asp Phe Leu Asp
200                 205                 210                 215

TCC AGG GAG GCT CCC CGG GAC ATC CAG GCG AGA ATG CGG ATC CAC AAC  1024
Ser Arg Glu Ala Pro Arg Asp Ile Gln Ala Arg Met Arg Ile His Asn
                220                 225                 230

AAC AGG GTG GGA CGC CAG GTG GTA ACG GAA AAC CTG AAG CGG AAG TGC  1072
Asn Arg Val Gly Arg Gln Val Val Thr Glu Asn Leu Lys Arg Lys Cys
            235                 240                 245

AAA TGC CAT GGA ACG TCA GGC AGC TGC CAA TTC AAG ACC TGT TGG AGG  1120
Lys Cys His Gly Thr Ser Gly Ser Cys Gln Phe Lys Thr Cys Trp Arg
        250                 255                 260

GCA GCG CCA GAG TTC CGG GCC ATC GGG GCA GCA CTG AGG GAG CGG CTG  1168
Ala Ala Pro Glu Phe Arg Ala Ile Gly Ala Ala Leu Arg Glu Arg Leu
    265                 270                 275

AGC AGA GCC ATC TTT ATC GAT ACC CAC AAC CGC AAC TCT GGA GCG TTC  1216
Ser Arg Ala Ile Phe Ile Asp Thr His Asn Arg Asn Ser Gly Ala Phe
280                 285                 290                 295
```

```
CAG CCC CGC CTA CGT CCG CGG CGC CTC TCT GGA GAG CTG GTT TAC TTT  1264
Gln Pro Arg Leu Arg Pro Arg Arg Leu Ser Gly Glu Leu Val Tyr Phe
                300                 305                 310

GAG AAG TCT CCT GAC TTC TGC GAG CGA GAC CCT ACT CTG GGC TCC CCA  1312
Glu Lys Ser Pro Asp Phe Cys Glu Arg Asp Pro Thr Leu Gly Ser Pro
            315                 320                 325

GGC ACG AGA GGC CGG GCT TGC AAC AAG ACC AGC CGC CTC TTG GAT GGC  1360
Gly Thr Arg Gly Arg Ala Cys Asn Lys Thr Ser Arg Leu Leu Asp Gly
        330                 335                 340

TGT GGC AGC CTG TGC TGT GGC CGT GGG CAC AAC GTG CTC CGG CAG ACG  1408
Cys Gly Ser Leu Cys Cys Gly Arg Gly His Asn Val Leu Arg Gln Thr
    345                 350                 355

CGA GTG GAG CGC TGC CAC TGT CGT TTC CAC TGG TGC TGT TAT GTG CTG  1456
Arg Val Glu Arg Cys His Cys Arg Phe His Trp Cys Cys Tyr Val Leu
360                 365                 370                 375

TGT GAT GAG TGT AAA GTC ACA GAG TGG GTC AAT GTG TGT AAA TGAAGGTGAG 1508
Cys Asp Glu Cys Lys Val Thr Glu Trp Val Asn Val Cys Lys
                380                 385

CCTCGCCTAG GCACGACGAG GAGGAGAAGC ACTGTGTGAG GGCTGCTCTC TTTCAGCCCT 1568

TTGCTCGGAT TTCTGTCTAG GGTTTATCGT GGCTCCCGGA AGCTCAGAGC ATCTGCCTGA 1628

GAACAGCTCT GGGGGTGTAG GGTCAGGTGA AATCTGTAAC GAGCAGCCTT TTGTGGGGGA 1688

AGTGGCCCCA CACTCTGTTC TTAAACACTC GAATAGACTA AGATGAAATG CACTGTACTG 1748

TTAGCGTCTT CTCTACCTAC AGCTCCCTCG GGCTCAGGTT CCTACTTCCT TTGGATAGGG 1808

AGTCTATCTT TTGGCCACTC CTCTTCCTCG AAGGATAATA GCAGGCATTG TGTGGAGTCA 1868

ATAAGACCCG TATATATAGC AAGAGACCAC CTCTTCCTAT TTGTGGTTCT CAAACTCCTC 1928

CACTACAGCC CAGAACCTCC TCTTATGGGA CCTCGGGTGA CAATAATGAG AGGTTTTCGG 1988

TTGGAAAAGG ACAGAGGGCA GGGAAGCCTC AGACAGCTGT CTTGTCAGGC TCTTGGGAGG 2048

CTTCTCCTTC CGTTCAGTTG TTGAAAGGGT CTCTCCAAAG GAAAGGTTTT AGCCATAACT 2108

CTTGGAGGCC CTTTTCCTTC TTCAGCAGGA AGGGTGGGAA TGGATAATTT ATTTTACTGA 2168

GATGTGTTCT TGGTTCCTGT TTGAAACTAA AATAAATTAA GTTACTGAAA AAAAAAAAAA 2228

AAAAA                                                           2233
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 389 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Met Leu Glu Glu Pro Arg Ser Arg Pro Pro Pro Leu Gly Leu Ala Gly
  1               5                  10                  15

Leu Leu Phe Leu Ala Leu Phe Ser Arg Ala Leu Ser Asn Glu Ile Leu
            20                  25                  30

Gly Leu Lys Leu Pro Gly Glu Pro Pro Leu Thr Ala Asn Thr Val Cys
        35                  40                  45

Leu Thr Leu Ser Gly Leu Ser Lys Arg Gln Leu Gly Leu Cys Leu Arg
    50                  55                  60

Ser Pro Asp Val Thr Ala Ser Ala Leu Gln Gly Leu His Ile Ala Val
65                  70                  75                  80

His Glu Cys Gln His Gln Leu Arg Asp Gln Arg Trp Asn Cys Ser Ala
                85                  90                  95

Leu Glu Gly Gly Gly Arg Leu Pro His His Ser Ala Ile Leu Lys Arg
            100                 105                 110

Gly Phe Arg Glu Ser Ala Phe Ser Phe Ser Met Leu Ala Ala Gly Val
        115                 120                 125

Met His Ala Val Ala Thr Ala Cys Ser Leu Gly Lys Leu Val Ser Cys
    130                 135                 140

Gly Cys Gly Trp Lys Gly Ser Gly Glu Gln Asp Arg Leu Arg Ala Lys
145                 150                 155                 160

Leu Leu Gln Leu Gln Ala Leu Ser Arg Gly Lys Thr Phe Pro Ile Ser
                165                 170                 175

Gln Pro Ser Pro Val Pro Gly Ser Val Pro Ser Pro Gly Pro Gln Asp
            180                 185                 190

Thr Trp Glu Trp Gly Gly Cys Asn His Asp Met Asp Phe Gly Glu Lys
        195                 200                 205

Phe Ser Arg Asp Phe Leu Asp Ser Arg Glu Ala Pro Arg Asp Ile Gln
    210                 215                 220

Ala Arg Met Arg Ile His Asn Asn Arg Val Gly Arg Gln Val Val Thr
225                 230                 235                 240

Glu Asn Leu Lys Arg Lys Cys Lys Cys His Gly Thr Ser Gly Ser Cys
                245                 250                 255
```

```
Gln Phe Lys Thr Cys Trp Arg Ala Ala Pro Glu Phe Arg Ala Ile Gly
            260                 265                 270

Ala Ala Leu Arg Glu Arg Leu Ser Arg Ala Ile Phe Ile Asp Thr His
        275                 280                 285

Asn Arg Asn Ser Gly Ala Phe Gln Pro Arg Leu Arg Pro Arg Arg Leu
    290                 295                 300

Ser Gly Glu Leu Val Tyr Phe Glu Lys Ser Pro Asp Phe Cys Glu Arg
305                 310                 315                 320

Asp Pro Thr Leu Gly Ser Pro Gly Thr Arg Gly Arg Ala Cys Asn Lys
                325                 330                 335

Thr Ser Arg Leu Leu Asp Gly Cys Gly Ser Leu Cys Cys Gly Arg Gly
            340                 345                 350

His Asn Val Leu Arg Gln Thr Arg Val Glu Arg Cys His Cys Arg Phe
        355                 360                 365

His Trp Cys Cys Tyr Val Leu Cys Asp Glu Cys Lys Val Thr Glu Trp
    370                 375                 380

Val Asn Val Cys Lys
385
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WHAT IS CLAIMED IS:

1. An isolated nucleic acid which comprises a sequence defined by SEQ ID NO: 16, or which hybridizes to such sequence under stringent conditions.

2. A nucleic acid according to claim 1 which encodes an HR2 polypeptide that is capable of inhibiting differentiation of a hematopoietic progenitor cell.

3. An isolated polypeptide capable of regulating hematopoietic progenitor cell development which comprises amino acid sequences defined by SEQ ID NOs: 1-9 or an amino acid sequence defined by SEQ ID NO: 17.

4. A polypeptide according to claim 3 which is an HR2 polypeptide capable of inhibiting differentiation of a hematopoietic progenitor cell.

5. An antibody which specifically binds to a polypeptide of either claim 3 or claim 4.

6. A method for modulating cellular development comprising contacting a hematopoietic progenitor cell with a compound having the biological activity of an HR1 or HR2 polypeptide, or with an agonist or antagonist of such a compound.

7. A method according to claim 6 in which the compound is an HR2 polypeptide that inhibits differentiation of the hematopoietic progenitor cell.

8. A pharmaceutical composition for regulating hematopoietic progenitor cell development comprising a pharmaceutically acceptable carrier and a compound having the biological activity of an HR1 or HR2 polypeptide, or an agonist or antagonist of such a compound.

9. A pharmaceutical composition according to claim 8 in which the compound is an HR2 polypeptide capable of inhibiting differentiation of a hematopoietic progenitor cell.

10. A pharmaceutical composition according to either claim 8 or 9 in which the compound is an HR2 polypeptide comprising an amino acid sequence defined by SEQ ID NO: 17.

11. A method for the manufacture of a pharmaceutical composition for regulating hematopoietic progenitor cell development comprising admixing a pharmaceutically acceptable carrier with a compound having the biological activity of an HR1 or HR2 polypeptide, or with an agonist or antagonist of such a compound.

12. The use of an HR1 or HR2 polypeptide for regulating hematopoietic progenitor cell development.

13. The use of an HR1 or HR2 polypeptide for the preparation of a medicament for regulating hematopoietic progenitor cell development.

14. The use of either claim 12 or 13 in which the polypeptide comprises an amino acid sequence defined by SEQ ID NO: 17.