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(54) Title
METHOD OF TRANSCRIPTIONALLY MODULATING GENE EXPRESSION AND OF DISCOVERING CHEMICALS CAPABLE OF FUNCTIONING AS GENE EXPRESSION MODULATORS

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(57) Claim

1. A method of transcriptionally modulating the expression of a gene of interest which encodes a protein, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism, which includes contacting a eukaryotic cell, which is capable of expressing the gene, with a molecule at a concentration effective to specifically transcriptionally modulate expression of the gene and thereby affect the level of the protein encoded by the gene of interest which is expressed by the cell, which molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene of interest, and (c) binds to DNA or RNA, binds to a protein through a domain of such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological effect.

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(54) Title: METHOD OF TRANSCRIPTIONALLY MODULATING GENE EXPRESSION AND OF DISCOVERING CHEMICALS CAPABLE OF FUNCTIONING AS GENE EXPRESSION MODULATORS

(57) Abstract

The present invention provides a method of transcriptionally modulating the expression of a gene-of-interest. The method comprises contacting a cell which is capable of expressing the gene with an amount of a molecule effective to transcriptionally modulate expression of the gene and thereby affect the level of the protein encoded by the gene which is expressed by the cell. Molecules useful in the practice of the invention are characterized as follows: (a) do not naturally occur in the cell, (b) bind to DNA or RNA or bind to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell. Additionally, this invention provides a method for determining whether a molecule known to be a modulator of protein biosynthesis is capable of transcriptionally modulating expression of a gene-of-interest.

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METHOD OF TRANSCRIPTIONALLY MODULATING GENE EXPRESSION AND OF DISCOVERING CHEMICALS CAPABLE OF FUNCTIONING AS GENE EXPRESSION MODULATORS

Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The expression of a specific gene can be regulated at any step in the process of producing an active protein. Modulation of total protein activity may occur via transcriptional, transcript-processing, translational or post-translational mechanisms. Transcription may be modulated by altering the rate of transcriptional initiation or the progression of RNA polymerase (28). Transcript-processing may be influenced by circumstances such as the pattern of RNA splicing, the rate of mRNA transport to the cytoplasm or mRNA stability. This invention concerns the use of molecules which act by

modulating the in vivo concentration of their target proteins via regulating gene transcription. The functional properties of these chemicals are distinct from previously described molecules which also affect gene transcription.

Researchers have documented the regulation of transcription in bacteria by low molecular weight chemicals (42, 36). Extracellular xenobiotics, amino acids and sugars have been reported to interact directly with an intracellular proteinaceous transcriptional activator or repressor to affect the transcription of specific genes.

Transcriptional regulation is sufficiently different between procaryotic and eucaryotic organisms so that a direct comparison cannot readily be made. Procaryotic cells lack a distinct membrane bound nuclear compartment. The structure and organization of procaryotic DNA elements responsible for initiation of transcription differ markedly from those of eucaryotic cells.

The eucaryotic transcriptional unit is much more complex than its procaryotic counterpart and consists of additional elements which are not found in bacteria. Eucaryotic transcriptional units include enhancers and other cis-acting DNA sequences (30, 19). Procaryotic transcription factors most commonly exhibit a "helix-turn-helix" motif in the DNA binding domain of the protein (29, 37). Eucaryotic transcriptional factors frequently contain a "zinc finger" (37, 12) or a "leucine zipper" (24) in addition to sometimes possessing the "helix-turn-helix" motif (26). Furthermore, several critical mechanisms at the post-transcriptional level such as RNA splicing and polyadenylation are not found in

procaryotic systems (21, 35).

In higher eucaryotes, modulation of gene transcription in response to extracellular factors can be regulated in both a temporal and tissue specific manner (22). For example, extracellular factors can exert their effects by directly or indirectly activating or inhibiting transcription factors (22, 28).

Modulators of transcription factors involved in direct regulation of gene expression have been described, and include those extracellular chemicals entering the cell passively and binding with high affinity to their receptor-transcription factors. This class of direct transcriptional modulators include steroid hormones and their analogs, thyroid hormones, retinoic acid, vitamin D_3 and its derivatives, and dioxins, a chemical family of polycyclic aromatic hydrocarbons (12, 38, 9).

Dioxins are molecules generally known to modulate transcription, however, dioxins bind to naturally-occurring receptors which respond normally to xenobiotic agents via transcriptionally activating the expression of cytochrome P450, part of an enzyme involved in detoxification. Similarly, plants also have naturally occurring receptors to xenobiotics to induce defense pathways. For example, the fungal pathogen Phytophthora megasperma induces an anti-fungal compound in soybeans. Such molecules which bind to the ligand binding domains of such naturally occurring receptors are not included on the scope of this invention.

The clinical use of steroid hormones, thyroid hormones, vitamin D_3 and their analogs demonstrates that agents

which modulate gene transcription can be used for beneficial effects, although these agents can exhibit significant adverse side effects. Obviously, analogs of these agents could have similar clinical utility as their naturally occurring counterparts by binding to the same ligand binding domain of such receptors.

Indirect transcriptional regulation involves one or more signal transduction mechanisms. The regulation typically involves interaction with a receptor, the receptor being part of a multistep intracellular signaling pathway, the pathway ultimately modulating the activity of nuclear transcription factors. This class of indirect transcriptional modulators include polypeptide growth factors such as platelet-derived growth factor, epidermal growth factor, cyclic nucleotide analogs, and mitogenic tumor promoters (18, 1, 2).

It is well documented that a large number of chemicals, both organic and inorganic, e.g. metal ions, can non-specifically modulate transcription.

Researchers have used nucleotide analogs in methods to modulate transcription. The mechanism involves incorporating nucleotide analogs into nascent mRNA or non-specifically blocking mRNA synthesis. Similarly, researchers have used alkylating agents, e.g. cyclophosphamide, or intercalating agents, e.g. doxorubicin, to non-specifically inhibit transcription.

Moreover, chemical inhibitors of hydroxymethyl-glutaryl CoA reductase, e.g. lovastatin, are known to modulate transcription by indirectly increasing expression of hepatic low density lipoprotein receptors as a consequence of lowered cholesterol levels.

Signal effector type molecules such as cyclic AMP, diacylglycerol, and their analogs are known to non-specifically regulate transcription by acting as part of a multistep protein kinase cascade reaction. These signal effector type molecules bind to domains on proteins which are thus subject to normal physiological regulation by low molecular weight ligands (10, 39).

The specific use of sterol regulatory elements from the LDL receptor gene to control expression of a reporter gene has recently been documented in PCT/US88/10095. One aspect of PCT/US88/10095 deals with the use of specific sterol regulatory elements coupled to a reporter as a means to screen for drugs capable of stimulating cells to synthesize the LDL receptor. PCT/US88/10095 describes neither the concept of simultaneously screening large numbers of chemicals against multiple target genes nor the existence of transcriptional modulators which (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the gene-of-interest, and (c) bind to DNA or RNA or bind to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand binding domain is normally associated with the defined physiological effect. The main focus of PCT/US88/10095 is the use of the sterol regulatory elements from the LDL receptor as a means to inhibit expression of toxic recombinant biologicals.

The use of molecules to specifically modulate transcription of a gene-of-interest as described herein has not previously been reported and its use will bring surprise since available literature does not propose the use of a molecule, as described, in a method to

specifically modulate transcription. Instead, the available literature has reported methods which define domains of transcriptional regulating elements of a gene-of-interest.

Further, the practice of using a reporter gene to analyze nucleotide sequences which regulate transcription of a gene-of-interest is well documented. The demonstrated utility of a reporter gene is in its ability to define domains of transcriptional regulatory elements of a gene-of-interest. Reporter genes which express proteins, e.g. luciferase, are widely utilized in such studies. Luciferases expressed by the North American firefly, Photinus pyralis and the bacterium, Vibrio fischeri were first described as transcriptional reporters in 1985 (8, 11)

A method to define domains of transcriptional regulating elements of a gene-of-interest typically has also involved use of phorbol esters, cyclic nucleotide analogs, concanavalin A, or steroids, molecules which are commonly known as transcriptional modulators. However, available literature shows that researchers have not considered using a transcription screen to identify specific transcriptional modulators. Apparently, success would be unlikely in doing so, however, we have demonstrated herein that this is not the case.

There is utility in developing the method of transcriptional modulation of a gene-of-interest by using such molecule as described herein. This method will allow the development of novel pharmaceuticals and circumvent many of the problems associated with the therapeutic use of recombinant biological factors.

Problems associated with the therapeutic use of

recombinant biological factors include the technical difficulties of large scale protein purification, the high costs of protein production, the limited shelf-life of most proteins and in some cases a short biological half-life of the administered protein in the organism. Additionally, therapeutic delivery of proteins normally requires injection and frequently induces an immune reaction in situations where chronic administration is required. The method described herein provides a means of up-regulating the expression of proteins, e.g. membrane receptors, which are not readily amenable to administration as injectable biologicals.

Furthermore, chemical molecules specifically regulating the activity of one member of a group of closely related proteins are difficult to produce. Molecules, structurally related at the protein level, may possess distinct regulatory elements at the DNA level which control their expression. Thus, molecules such as the chemical transcriptional modulators defined herein can provide a greater opportunity for specifically modulating the activity of structurally related proteins. One example is the ras oncogene family, where the H-, N- and K-ras proteins are highly related but wherein the three genes have distinct structures.

Finally, the molecules described herein may also serve to mimic normal physiological response mechanisms, typically involving the coordinated expression of one or more groups of functionally related genes. Therefore, determining whether a molecule can specifically transcriptionally modulate the expression of a gene-of-interest and the ultimate clinical use of the molecule provides a therapeutic advantage over the use of single recombinant biologicals, or drugs which bind directly to the final target protein encoded by the gene-of-interest.

summary of the Invention.

The present invention provides a method of transcriptionally modulating the expression of a gene-of-interest, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. The method comprises contacting a cell which is capable of expressing the gene with an amount of a molecule effective to transcriptionally modulate expression of the gene and thereby affect the level of the protein encoded by the gene which is expressed by the cell.

Accordingly, the present invention provides a method of transcriptionally modulating the expression of a gene of interest which encodes a protein, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism, which includes contacting a eukaryotic cell, which is capable of expressing the gene, with a molecule at a concentration effective to specifically transcriptionally modulate expression of the gene and thereby affect the level of the protein encoded by the gene of interest which is expressed by the cell, which molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene of interest, and (c) binds to DNA or RNA, binds to a protein through a domain of such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological effect.

Molecules useful in the practice of the invention are characterized as follows (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the gene-of-interest, and (c) bind to DNA or RNA or bind to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand binding domain is normally associated with the defined physiological or pathological effect.

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Additionally, this invention provides a method of determining whether a molecule, not previously known to be a modulator of protein biosynthesis, is capable of transcriptionally modulating the expression of a gene-of-interest. The method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each such cell comprises DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene-of-interest, (ii) a promoter of the gene-of-interest, and (iii) a reporter gene which expresses a

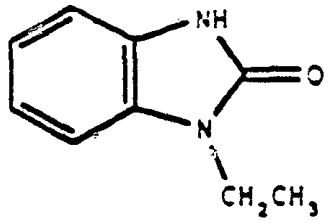
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polypeptide capable of producing a detectable signal, coupled to, and under the control of, the promoter, and the contacting is carried out under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene-of-interest, causes a measurable detectable signal to be produced by the polypeptide expressed by the reporter gene and the amount of the signal produced may be quantitatively determined. The amount of produced signal so determined is compared with the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule so as to thereby identify the molecule as one which causes a change in the detectable signal produced by the polypeptide expressed by the reporter gene and thus identify the molecule as a molecule capable of transcriptionally modulating the expression of the gene-of-interest.

The present invention still further provides a method for transcriptionally modulating in a multicellular organism the expression of a gene-of-interest, the expression of which is associated with a defined physiological or pathological effect in the organism. The method comprises administering to the organism an amount of a molecule effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect. A molecule useful in the method: (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene-of-interest, and (c) binds to DNA or RNA or binds to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the organism, the binding of a ligand to which ligand binding domain is normally associated with the defined physiological or

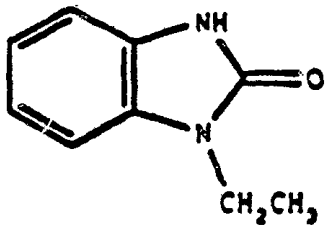
pathological effect.

This invention provides a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:



effective to enhance the expression of human growth hormone by the cell.

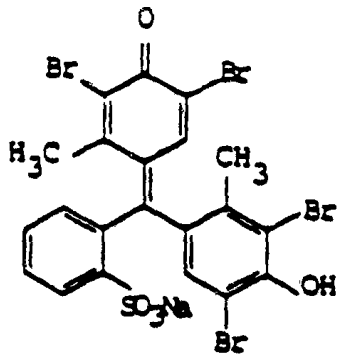
Additionally, this invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:



effective to enhance expression of human growth hormone by, and thus growth of, the human being.

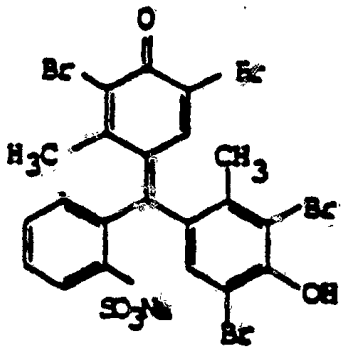
The invention also provides a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of

expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:



effective to enhance the expression of human growth hormone by the cell.

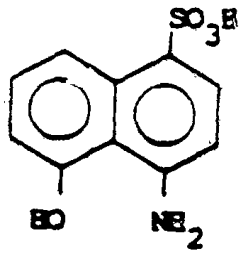
Another provision of the present invention concerns a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:



effective to enhance expression of human growth hormone by, and thus growth of, the human being.

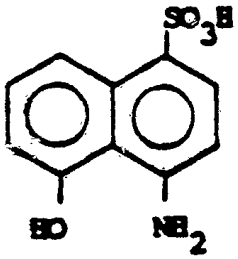
This invention also concerns a method for enhancing the expression of human growth hormone by a cell which (i)

comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:



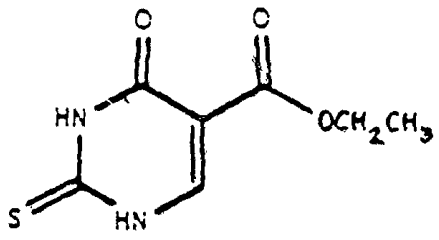
effective to enhance the expression of human growth hormone by the cell.

Further, this invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:



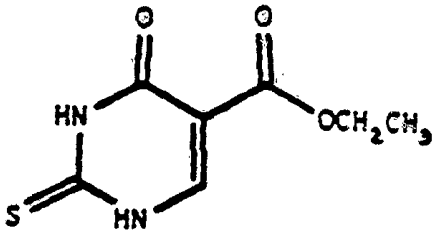
effective to enhance expression of human growth hormone by, and thus growth of, the human being.

Additionally, this invention concerns a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:



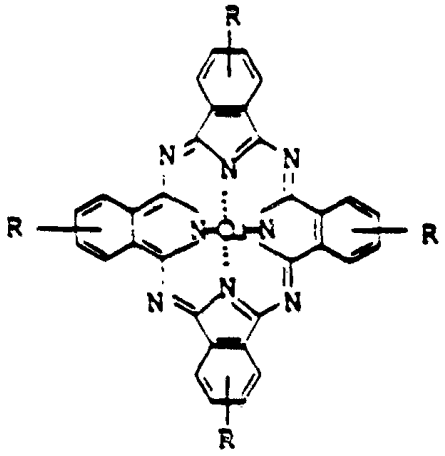
effective to enhance the expression of human growth hormone by the cell.

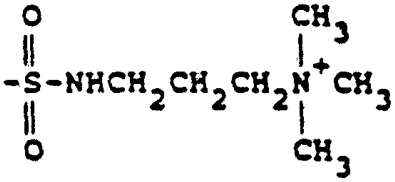
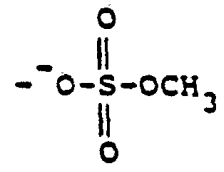
Additionally, the invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:



effective to enhance expression of human growth hormone by, and thus growth of, the human being.

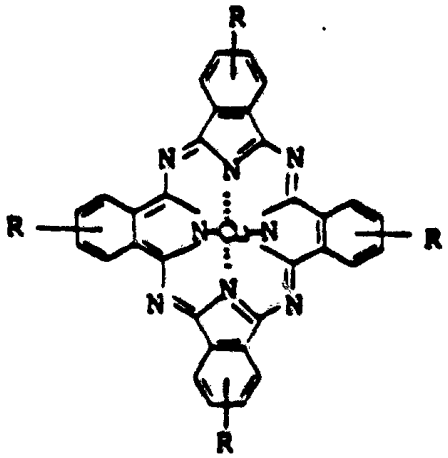
Further, the invention provides another method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:

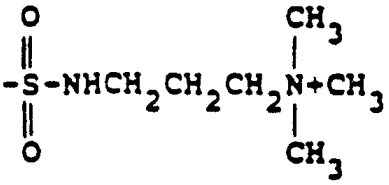
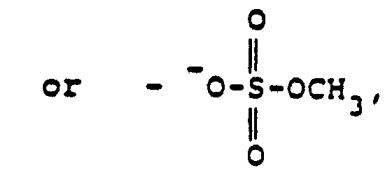


wherein R is  or ,

effective to enhance the expression of human growth hormone by the cell.

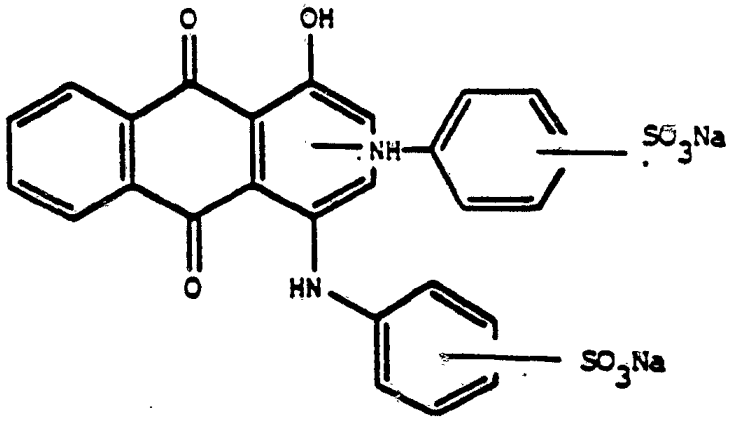
Additionally, the invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:



wherein R is  or ,

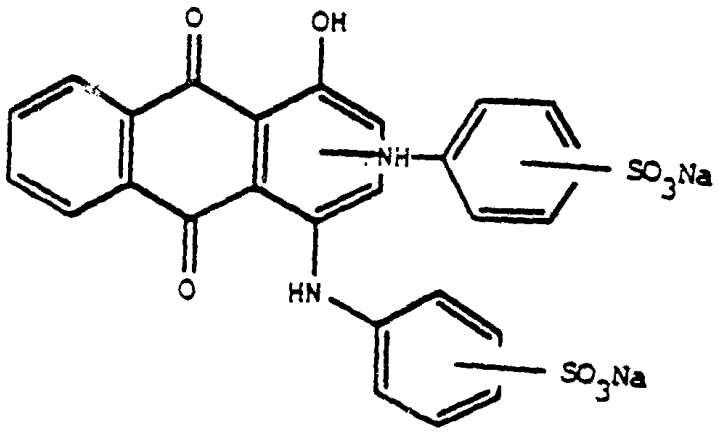
effective to enhance expression of human growth hormone by, and thus growth of, the human being.

Further, this invention provides a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:



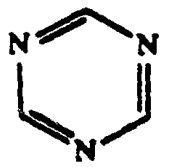
effective to enhance the expression of human growth hormone by the cell.

Further more, this invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:



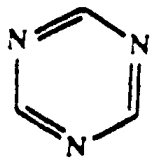
effective to enhance expression of human growth hormone by, and thus growth of, the human being.

The present invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



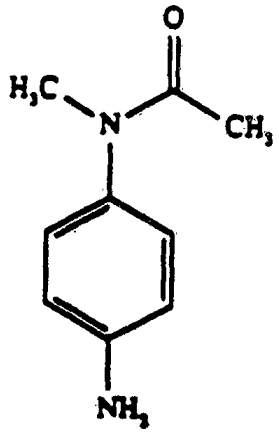
effective to enhance the expression of G-CSF by the cell.

The present invention also provides a method for increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:



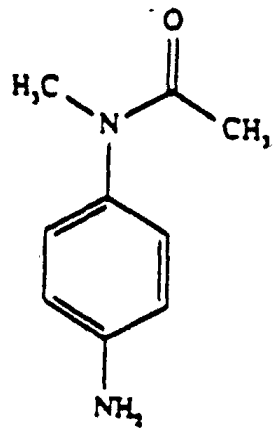
effective to enhance the expression of GCSF by, and thus increase the formation of neutrophils in, the human being.

The present invention also provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



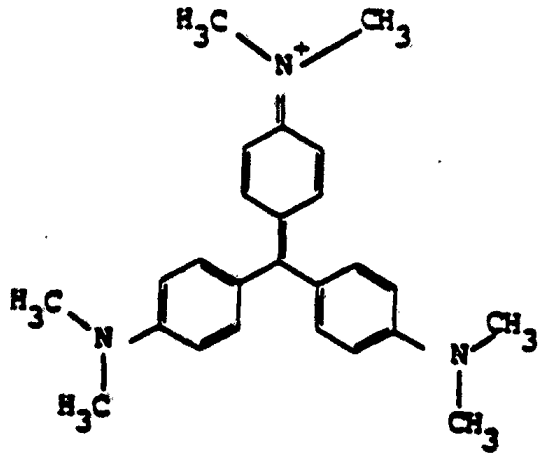
effective to enhance the expression of G-CSF by the cell.

Additionally, the invention provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:



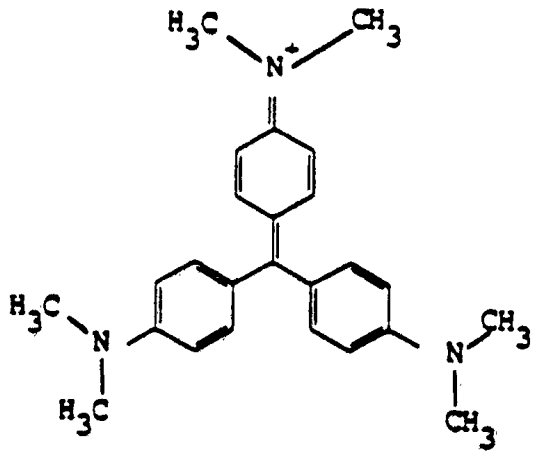
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Further, the invention provides another method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



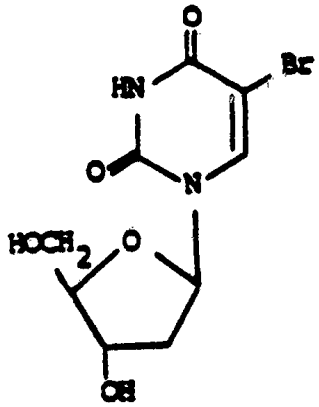
effective to enhance the expression of G-CSF by the cell.

The present invention provides another method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:



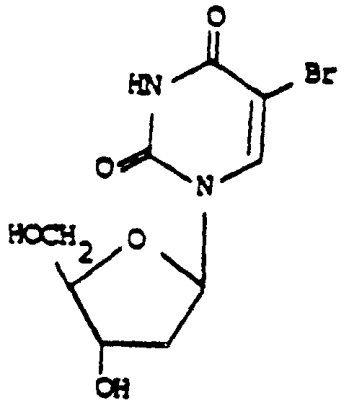
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

The invention also concerns a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF comprising contacting the cell with an amount of a molecule having the structure:



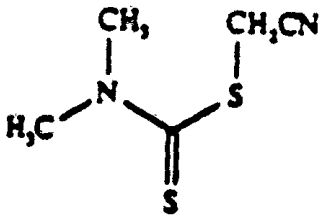
effective to enhance the expression of G-CSF by the cell.

Additionally, the invention concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



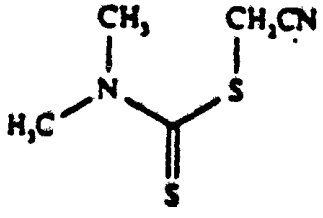
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

This invention further concerns a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



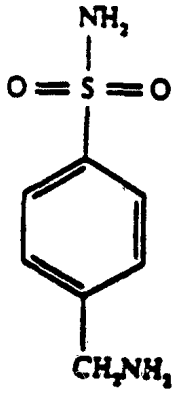
effective to enhance the expression of G-CSF by the cell.

Further the invention provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



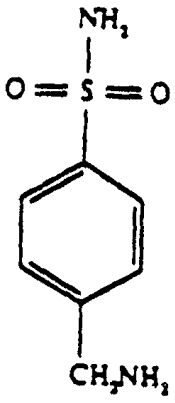
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Additionally, the present invention concerns a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising contacting the cell with an amount of a molecule having the structure:



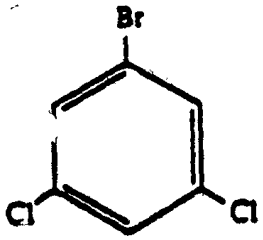
effective to enhance the expression of G-CSF by the cell.

The invention further concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF wherein the method comprises administering to the human being an amount of a molecule having the structure:



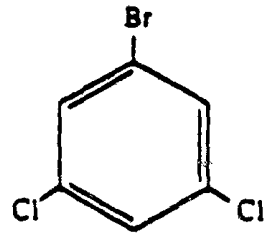
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Also, the invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



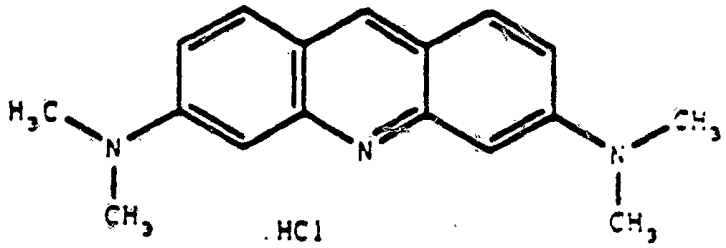
effective to enhance the expression of G-CSF by the cell.

The invention additionally concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, wherein the method comprises administering to the human being an amount of a molecule having the structure:



effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in the human being.

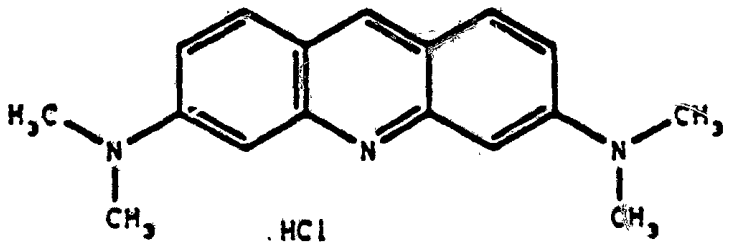
Another provision of the present invention is a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



.1/2 ZnCl2

effective to enhance the expression of G-CSF by the cell.

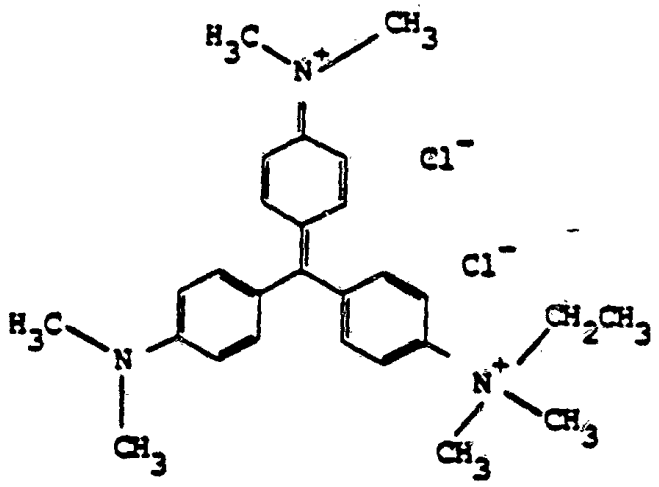
Additionally, the invention provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:



1/2 ZnCl2

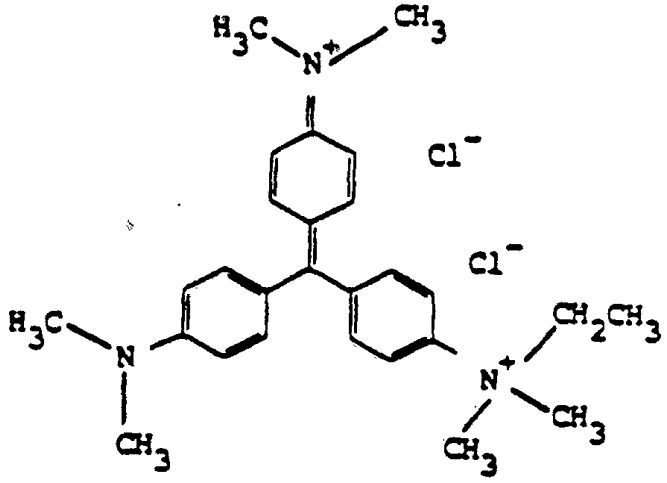
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in the human being.

The present invention provides another method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



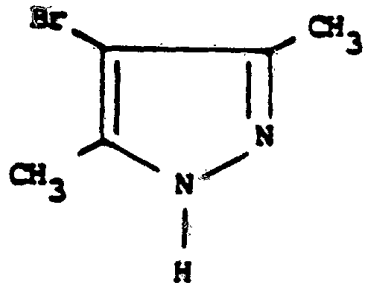
effective to enhance the expression of G-CSF by the cell.

Further, the present invention concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:



effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

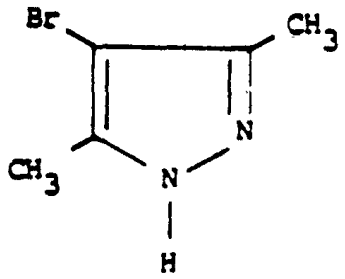
This invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the cell.

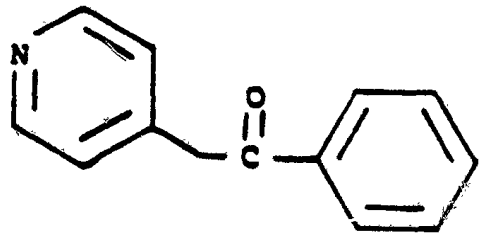
Additionally, this invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises

DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



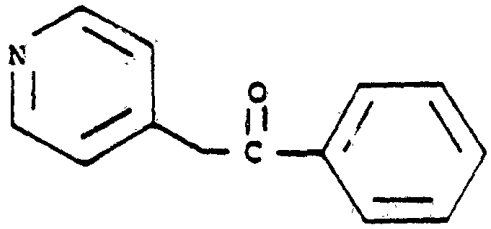
effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

Further, the invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



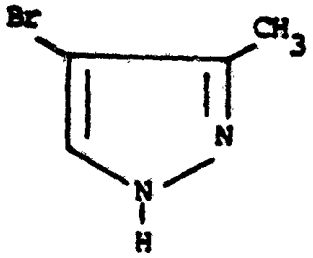
effective to decrease the expression of G-CSF by the cell.

Additionally, this invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



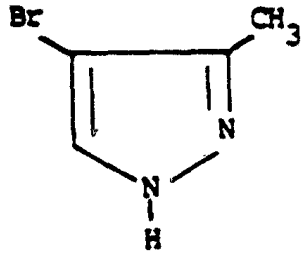
effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

The invention further provides a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



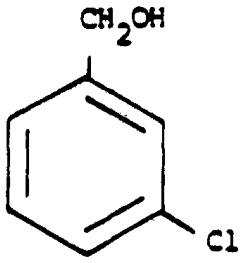
effective to decrease the expression of G-CSF by the cell.

Additionally, this invention provides a method of decreasing the formation of neutrophils and effecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



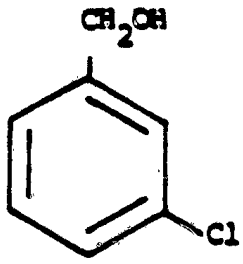
effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

Further, the present invention a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the cell.

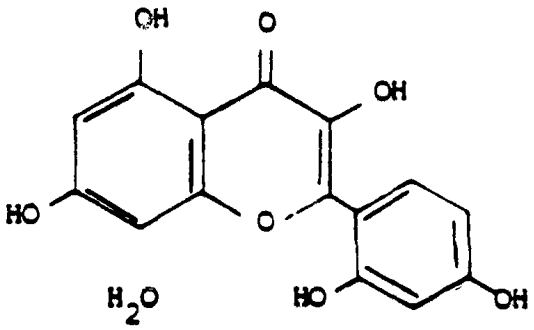
Additionally, this invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

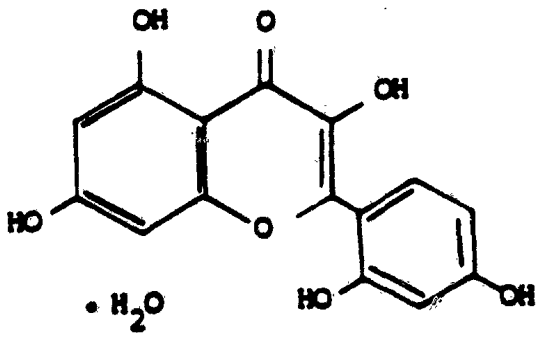
The present invention further provides a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of

expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the cell.

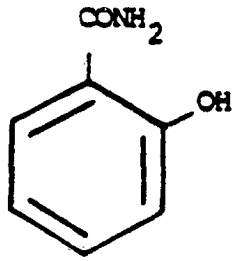
Further still the invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic

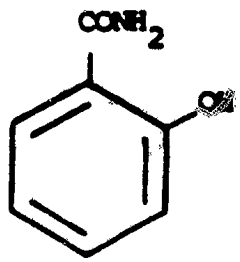
functions of neutrophils in, the human being.

The present invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



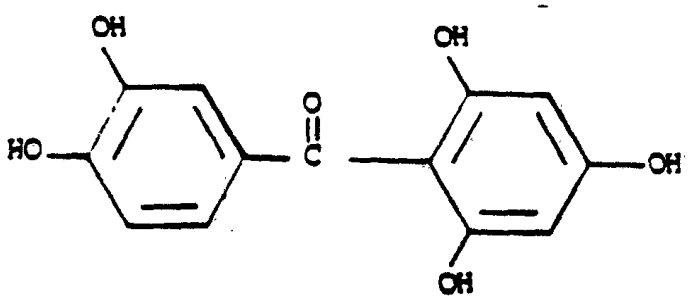
effective to decrease the expression of G-CSF by the cell.

Additionally, the present invention concerns a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



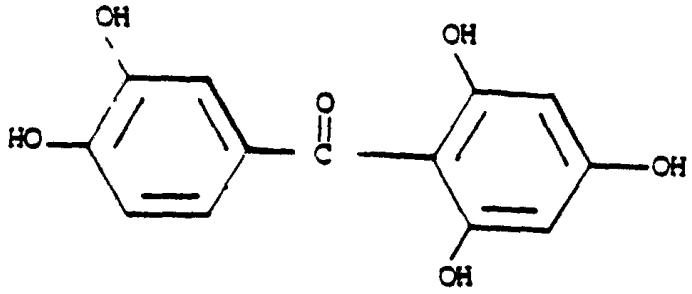
effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

The present invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



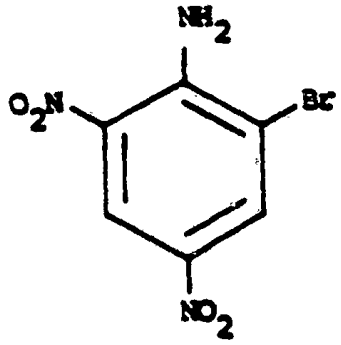
effective to decrease the expression of G-CSF by the cell.

Further, the invention concerns a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

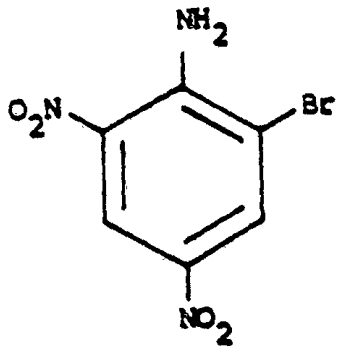
Moreover, the invention provides a method of decreasing the expression of a mammary tumor virus by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, a mammary tumor virus. The method comprises contacting the cell with an amount of a molecule having the structure:



effective to decrease the expression of a mammary tumor virus by the cell.

Finally, the invention also provides a method of suppressing the proliferation of a mammary tumor virus in a subject who (i) comprises DNA encoding, and (ii) is capable of expressing, a mammary tumor virus,

comprising administering to the subject an amount of a molecule having the structure:



effective to suppress the proliferation of a mammary tumor virus in the subject.

Brief Description of the Figures

Figure 1 is a partial restriction enzyme cleavage map of the plasmid pD0432 which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 2 is a partial restriction enzyme cleavage map of the plasmid pSVLuci which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 3 is a partial restriction enzyme cleavage map of the plasmid pMLuci which contains the luciferase gene of the firefly, Photinus pyralis and the mouse mammary tumor virus long terminal repeat.

Figure 4 is a partial restriction enzyme cleavage map of the plasmid pUXLuci which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 5 is a partial restriction enzyme cleavage map of the plasmid phGH:CAT which contains the CAT gene and human growth hormone promoter sequences.

Figure 6 is a partial restriction enzyme cleavage map of the plasmid phGH-Luci which contains the luciferase gene from the firefly, Photinus pyralis and human growth hormone promoter sequences.

Figure 7 is a partial restriction enzyme cleavage map of the plasmid pJM710 which contains G-CSF upstream sequences.

Figure 8 is a partial restriction enzyme cleavage map of the plasmid pGEM5-Luci which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 9 is a partial restriction enzyme cleavage map of the plasmid PG-Luc 1 which contains both the luciferase gene from the firefly, Photinus pyralis, and G-CSF upstream sequences.

Figure 10 is a bar graph illustrating induction of luciferase expression in reporter cell lines containing MMTV (M10), human growth hormone (532), and human G-CSF (G21) promoter sequences, in response to known transcriptional inducers.

Figure 11 is a bar graph illustrating the effect of steroids on luciferase expression in the MMTV reporter cell line M10.

Figure 12 is a bar graph illustrating specific induction of luciferase expression in reporter cell lines for MMTV (M10), human growth hormone (532) and human G-CSF (G21) promoters in response to chemicals identified in a high throughput screen and known transcriptional inducers.

Figure 13 is a bar graph illustrating specific inhibition of luciferase expression in reportor cell lines for MMTV (M10), human growth hormone (532), and human G-CSF (G21) in response to chemicals identified in a high throughput screen.

Figure 14 is an autoradiograph of a Northern blot illustrating increased G-CSF mRNA production by the human epithelial cell line U5637 in response to chemicals #670 and #1255 and IFN-gamma as compared to the solvent DMSO. Reprobing with beta-actin was used to normalize for the amount of mRNA that had been loaded onto the gel.

Figure 15 is an autoradiograph of a polyacrylamide gel

illustrating an S1 nuclease protection analysis of increased mRNA production by the human bladder carcinoma cell line 5637 in response to lead chemicals #542, #1255, #1793 and #1904. "RNA" indicates the sources of the RNA preparations used in individual lanes. "Probe" indicates the mRNA-specificities of probes used in individual lanes. "Compound" lists the compounds with which the 5637 cells were treated prior to RNA extraction and loading on individual gel lanes ("Cyclo" means cycloheximide). "Conc" indicates three different compound concentrations used in the experiment (L = low, M = medium, H = high). G, GM and A indicate the correct sizes of G-CSF-, GM-CSF- and Actin-specific nuclease-protected mRNA/Probe hybrids.

Figure 16 illustrates a dose response analysis of chemicals #80, #670, and #1780 using the G-CSF reporter cell line G21. The amount of luciferase expression is indicated in arbitrary units.

Figure 17 is a bar graph illustrating increased G-CSF secretion by 5637 cells treated for 48 hours in serum-containing media with the samples indicated on the abscissa. TNF-alpha was used at 5 ng/ml. Chemicals #542 and #1780 were used at 50 uM or 1 uM and 0.2 uM final concentration, respectively. Both chemicals were used in DMSO at a final concentration of 0.5 %. The ordinate indicates the concentration of G-CSF secreted into 5 ml of serum-containing media by 25 square cm of confluent 5637 cells.

Figure 18 illustrates a dose response analysis of chemical #542 using the G-CSF reporter cell line G 21 (solid line) and the MTT respiratory inhibition cytotoxicity assay (dotted line). Respiratory inhibition in percent of untreated control cells (Ordinate, left

scale) and luciferase expression of #542- treated over solvent-treated cells (ordinate, right scale) are plotted against #542 concentration (abscissa).

Detailed Description of the Invention

The present invention provides a method of transcriptionally modulating the expression of a homologous gene-of-interest, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. The method comprises contacting a cell which is capable of expressing the gene with an amount of a molecule effective to transcriptionally modulate the expression of the gene. Modulating the expression of the gene affects the level of the protein encoded by the gene which is expressed by the cell. As used herein a homologous gene-of-interest means a gene which is naturally associated with an organism or virus encoding and expressing the gene. Therefore, this definition would not include a synthetic or recombinant gene constructed by genetic engineering methods so as to position the gene-of-interest under the control of a heterologous promoter.

The term "physiological effect" as used herein is defined as an effect characteristic of, or appropriate to, an organism's healthy or normal functioning. Further, the term "pathological effect" as used herein is defined as an effect altered or caused by disease.

Molecules useful in the practice of the invention are characterized as follows (a) do not naturally occur in the cell, (b) specifically transcriptionally modulate expression of the gene-of-interest, and (c) bind to DNA or RNA or bind to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand binding domain is normally

associated with the defined physiological or pathological effect.

In one example of this invention, the molecule would not occur naturally in any cell of a higher eucaryotic organism. In another example of this invention the molecule would not occur naturally in any cell, e.g. a mineral. In yet another example, the molecule would not occur naturally, e.g. a synthetic molecule.

The phrase "specifically transcriptionally modulate expression of the gene-of-interest" as used herein means modulating the expression of the gene-of-interest without modulating the expression of other genes in the cell in a way which would cause an adverse effect on (a) an organism containing the cell in the case where the cell is within the organism or (b) the growth or the culturing of the cell, in the case where the cell is being grown or cultured to make a product where the amount of product produced is associated with expression of the gene-of-interest. However, within this definition where the drug is used to treat, for example, parasitic infection, drug application is intended to cause an adverse effect on the cells of the parasite (which may contain the gene-of-interest), but not on the cells of the host organism. In this context, a gene-of-interest may constitute a single gene or a limited number of genes whose expression can be functionally coordinated. One example of coordinate gene regulation is exhibited by the physiological growth modulators termed the transforming growth factor-β family of polypeptides (TGF-βs) (90). TGF-βs control the extracellular matrix (ECM) by; (1) increasing expression of the genes encoding ECM polypeptides, e.g. collagen, fibronectin and osteopontin; (2) increasing expression of receptors for the ECM, e.g.

the integrins; and (3) increasing expression of protease inhibitors (e.g. TIMP and PAI-I) while decreasing expression of secreted proteases (e.g. collagenase and stromelysin). This coordinate regulation may allow TGF-βs to be useful in the repair of surface wounds, cartilage and bone. Molecules with properties as described in this invention, with lower molecular weights than TGF-β, and which can be either chemically synthesized or readily isolated from natural sources, yet mimic the coordinate regulation of extracellular matrix as induced by the TGF-βs, have significant advantages as therapeutic agents over the use of such complex polypeptides.

Moreover, the phrase "transcriptionally modulate the gene-of-interest" infers a notion of directness. Thus, as used herein, "transcriptionally modulate expression of a gene-of-interest" by a molecule means the effect upon transcription of the gene resulting from either (a) direct binding of the molecule to DNA or RNA, a DNA- or RNA-binding protein, and/or a DNA- or RNA- binding protein complex, or (b) direct binding of the molecule to a protein which directly chemically modifies a DNA- or RNA- binding protein or protein complex.

As used herein "chemically modifies" a DNA- or RNA-binding protein or protein complex means to modify the protein or protein complex through a chemical reaction, including but not limited to, phosphorylation, glycosylation, methylation, acetylation, adenoribosylation, acylation, myristylation, reduction, oxidation, covalent oligomerization or polymerization or proteolytic cleavage.

The invention provides a cell capable of expressing the

gene-of-interest, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism. The cell may be a human cell, an animal cell, a plant cell or any eucaryotic cell or procaryotic cell from whatever source.

Further, in the practice of the invention, the gene-of-interest whose expression is associated with a defined physiological or pathological effect within a multicellular organism, may be a human gene.

Moreover, the gene-of-interest may encode a hematopoietic protein. Hematopoietic proteins may include, but are not limited to, colony stimulating factors and erythropoietin (EPO).

Examples of colony stimulating factors useful in the practice of this invention include, but are not limited to, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and macrophage colony stimulating factor (M-CSF).

Further, the gene-of-interest of the invention may encode an interleukin (IL) or a cytokine, or a growth modulating factor. One example of such a growth modulating factor would be a member of the transforming growth factor-β (TGF-β) family i.e. TGF-β1 or TGF-β2 or TGF-β3. A gene-of-interest may also encode a receptor for a steroid hormone, such as the testosterone receptor or the estrogen receptor or a receptor for a TGF-β.

The gene-of-interest may also encode a growth hormone. Examples of growth hormones include, but are not limited to, human, bovine, porcine, avian, ovine, piscine, and

equine growth hormones. Additionally, the gene-of-interest may also encode polypeptide analogs of the above-identified growth hormones. Additionally, a gene-of-interest may encode a growth hormone releasing factor.

The present invention also provides a viral gene as the gene-of-interest. The viral gene may be a retroviral gene. Retroviral genes of the invention may be from the HIV, HTLV-1, or HTLV-2 virus.

In the practice of the invention the viral gene may be a gene from a hepatitis virus, a herpes virus, a papilloma virus, a cytomegalovirus, or an animal virus.

Animal viruses of the invention may include, but are not limited to, pseudorabies, Marek's, Newcastle's Disease, and IBR viruses.

The gene-of-interest, whose expression is associated with a defined physiological or pathological effect within a multicellular organism, may also be a plant gene. The plant gene may encode an agronomically important trait. Examples of agronomically important traits may include, but are not limited to, germination, sprouting, flowering, fruit ripening, salt tolerance, herbicide resistance, pesticide resistance, fungicide resistance, temperature resistance, and growth.

Additionally, in the practice of the invention the gene-of-interest may be a protozoan gene. Examples of protozoans may include, but are not limited to, a selection from the group consisting of Trypanosoma, Plasmodium, Leishmania, Giardia, Entamoeba, Toxoplasma, Babesia, and Cryptosporidiosis.

Moreover, the gene-of-interest whose expression is associated with a defined physiological or pathological effect within a multicellular organism, may be a helminth gene.

Further, the gene-of-interest may also be an oncogene. Examples of oncogenes may include, but are not limited to, the phl-abl oncogene, the neu oncogene, or the src oncogene. Additionally, the oncogene may be selected from the group consisting of H-ras, N-ras, and K-ras oncogenes.

The present invention additionally provides that the gene-of-interest, whose expression is associated with a defined physiological or pathological effect within a multicellular organism, may encode a naturally occurring receptor. The naturally occurring receptor may be the human low density lipoprotein (LDL) receptor. Further, the receptor may be the receptor for a hemopoietic protein. Examples of hematopoietic proteins may include, but are not limited to, a selection from the group consisting of M-CSF, G-CSF, GM-CSF, and EPO.

The naturally occurring receptor encoded by the gene-of-interest may also be the receptor for an interleukin (IL). Examples of an IL may include, but are not limited to, a selection from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 and IL-8.

Additionally, in the practice of the invention, the naturally occurring receptor may be a cell surface protein which mediates infection of the cell by a virus. Examples of viruses may include, but are not limited to, HIV, HTLV-1, HTLV-2, a hepatitis virus, a herpes virus, a papilloma virus, a cytomegalovirus and a rhinovirus.

In one example of the invention, the receptor which naturally occurs in the cell is a testosterone receptor. In another example of the invention, the receptor which naturally occurs in the cell is an estrogen receptor.

Typically, a ligand, in the context of this invention, is a molecule with a molecular weight of less than 5,000 daltons, more typically less than 2,000 daltons.

This invention also provides a method of determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene-of-interest. The method comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested. Each cell comprises DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene-of-interest, (ii) a promoter of the gene-of-interest, and (iii) a reporter gene which expresses a polypeptide capable of producing a detectable signal, coupled to, and under the control of, the promoter. The polypeptide expressed by the reporter gene produces the detectable signal under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene-of-interest, causes a measurable detectable signal to be produced by the polypeptide expressed by the reporter gene.

Quantitatively determining the amount of the signal produced requires comparing the amount of signal produced compared to the amount of produced signal detected in the absence of any molecule being tested or upon contacting the sample with any other molecule. The

comparison permits the identification of the molecule as one which causes a change in the detectable signal produced by the polypeptide expressed by the reporter gene and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene-of-interest.

The phrase "a modulatable transcriptional regulatory sequence of a gene-of-interest" as used herein concerns a DNA sequence capable of regulating the initiation of transcription from the promoter of the gene-of-interest.

Molecules useful in the practice of this invention have the following characteristics. The molecule does not naturally occur in the cell. The molecule specifically transcriptionally modulates expression of the gene-of-interest. Further, the molecule binds to DNA or RNA or binds to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell. The binding of a ligand to the ligand binding domain is normally associated with the defined physiological or pathological effect.

The term "promoter" of a gene-of-interest is defined herein as a minimal DNA sequence necessary for specific initiation of transcription.

In the practice of the invention the sample may comprise cells in monolayers or cells in suspension. The cells of the invention may comprise human, animal, or plant cells. In one example of the invention, the cells are bacterial cells. In another example of the invention, the cells are fungal cells.

Further, the invention provides that the predefined number of cells contained in the sample may be from about 2×10^2 to about 5×10^5 cells, typically from about 10^3 to about 5×10^4 cells. In theory, the ideal method might use only one cell. In practice, the method may include at least 10 cells per samples. More practically the method may include at least 100 cells per sample.

The invention also provides that the predetermined amount of the molecule to be tested may be based on the volume of the sample. Further, the predetermined amount of the molecule to be tested may be from about 1pM to about 500μM. Typically, in a primary highthroughtput screen the predetermined amount would be from about 10nM to about 500μM. Typically, in a secondary screen to evaluate initial lead compounds, the predetermined amount would be from about 1pM to about 20 μM.

Further, the invention provides that contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested may be effected from about 1 to about 24 hours, typically from about 2 to about 12 hours. Moreover, contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested may be effected with more than one predetermined amount of the molecule to be tested. The molecule to be tested may be, but is not limited to, a purified molecule. As used herein, the molecule may also be a mixture of different molecules, e.g. in a fermentation broth or in a natural product extract. Typically, a fermentation broth would be produced by fermentation of a suitable sample under standard conditions known to those skilled in the art. Examples of natural product extracts would include, but are not restricted to, plant extracts, seaweed extracts,

insect extracts, spider venoms, etc.

A minimal modulatable transcriptional regulatory sequence may comprise a few nucleotides in length, such as the so-called CCAAT box motif, the octamer binding motif or the heat shock element (91). A gene-of-interest may typically possess multiple motifs with different combinations of modulatable transcriptional regulatory sequences. It has been shown that enhancer elements or other regulatory motifs can be present at many kilobases upstream or downstream from the transcription start site of a gene-of-interest. Thus, as used herein, a modulatable transcriptional regulatory sequence may comprise a few nucleotides or may be naturally or synthetically constructed having multiple elements spanning several hundred kilobases in length. Transcription can also be influenced by the particular nuclear location or local chromatin configuration. In order to optimize a screen for substances that modulate regulatory elements, it may be preferable to situate a reporter gene at the exact chromosomal region where the gene-of-interest resides normally. However, it would be clear to one skilled in the art that the reporter gene may be situated anywhere in a chromosome. In one example in the invention, the modulatable transcriptional regulatory sequence comprises a cloned genomic regulatory sequence. In another example of the invention, the DNA consists essentially of more than one modulatable transcriptional regulatory sequence.

A series of experiments described by K.R. Thomas and M.R. Capecchi (44,45,46) have shown that it is possible to direct transfected or microinjected DNA, using homologous DNA sequences, to a specific chromosomal location in mammalian cells. Using homologous recombination, it is possible to replace a gene-of-interest with other

sequences at a high frequency. The materials required include a reporter gene (such as luciferase), a selectable marker (such as the neomycin phosphotransferase II gene from Tn5), and sequences derived from the gene-of-interest. These materials would be used to construct a vector which would include, in one embodiment of this invention, a fusion consisting of the gene-of-interest (free of its normal upstream promoter sequences) and the reporter gene where the reporter gene product is still active.

Two types of selectable markers, one positive and one negative, such as the Herpes Simplex thymidine kinase (TK), greatly enhance the likelihood of achieving homologous recombination. Use of one copy of the TK gene located outside the homologous regions at either end allows for selection of "correct" homologous recombinants. Neomycin resistant cells which are recombined randomly will usually insert via the ends of the linearized, transfected DNA and thus include the TK gene(s). This will allow for selection against these cells in the presence of gancyclovir which is converted to a toxic product. This is the method specifically described by Capecchi. However, this method has not been used to produce cell lines to screen for compounds which modulate the expression of a target gene-of-interest as described herein.

The invention provides that the reporter gene in the DNA contained in the cell sample, which expresses a polypeptide capable of producing a detectable signal coupled to, and under control of, the promoter, may be inserted downstream of the endogenous promoter of the gene-of-interest by homologous recombination. The following provides a method of determining whether a

molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene-of-interest which comprises contacting a sample which contains a predefined number of cells with a predetermined amount of a molecule to be tested, each such cell comprising DNA consisting essentially of (i) a modulatable transcriptional regulatory sequence of the gene-of-interest, (ii) a promoter of the gene-of-interest, and (iii) a DNA sequence transcribable into mRNA coupled to and under the control of, the promoter, under conditions such that the molecule, if capable of acting as a transcriptional modulator of the gene-of-interest, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence, quantitatively determining the amount of the mRNA produced, comparing the amount so determined with the amount of mRNA detected in the absence of any molecule being tested or upon contacting the sample with any other molecule, and thereby identifying the molecule as one which causes a change in the amount of detectable mRNA, and thus identifying the molecule as a molecule capable of transcriptionally modulating the expression of the gene-of-interest. In one example of the above-described method, the molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene-of-interest, and (c) binds to DNA or RNA or binds to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand binding domain is normally associated with a defined physiological or pathological effect.

Modulatable transcriptional regulatory sequences may also

occur within introns of a gene-of-interest. Typically, the exact location of all such regulatory sequences of a gene-of-interest will be unknown. In one example of this invention, the reporter gene will be a reporter gene which can be transcribed into mRNA which can be quantified as a detectable signal. This approach would allow (i) allow all the modulatable transcriptional regulatory sequences of the gene to be in their native environment and arrangement relative to the gene-of-interest including any and all transcriptional regulatory sequences within introns, (ii) obviate the necessity of using permanent cell lines to construct reporter cell lines and (iii) not require knowledge of promoter and modulatable transcriptional regulatory sequences.

In one example of the invention, mRNA is detected by quantitative polymerase chain reaction. Methods for direct detection of mRNA in a rapid and quantitative manner that fulfill the needs of a high throughput screening method described in this invention are not available. A suitable assay would have to meet the following criteria: (i) specific detection of fewer than 50,000 mRNA molecules, (ii) short assay time of a few hours, (iii) simple chemistry, that is amenable to automation. Ideally a "single tube" assay would be used which can be carried out within the same container in which the cells to be tested have been cultured, by eliminating transfer steps which may be time consuming and contribute to assay variation.

Several techniques have been described by others, which may be modified to be useful in the context of this invention. All assays are based on the principle of using a single stranded nucleotide probe containing sequences complementary to the target mRNA transcribed from the

gene-of-interest which probe is attached to a solid support to rapidly concentrate (capture) the mRNA out of a crude cell lysate. Cell lysates can be prepared such that ribonucleases are inhibited while hybridization of nucleic acids remains possible, for example in high concentrations of guanidinium isothiocyanate (47). Target mRNA bound to the solid phase can be freed of contaminants by intensive washing and subsequently detected with a second probe which is complementary to a different region of the target mRNA than the capture probe. The second probe would carry an appropriate detectable label.

To obtain optimal sensitivity i.e. obtain a maximal number of labels being specifically bound to a target molecule in the presence of a minimal number of labels being unspecifically retained by other components of the assay mixture, various techniques can be applied to increase signal to noise ratios. A procedure termed reversible target capture has been designed to reduce the background noise by capturing the target molecule - label complexes onto capture probes linked to solid phase, release of the complexes and transfer of the complexes onto new capture probes linked to solid phase and so forth, cycling between steps. Background is reduced by this procedure, because the release step is designed not to release non-specifically bound label that will not therefore be transferred together with the target molecules and will eventually be removed from the mixture (48). This method has a reported sensitivity of 15,000 target mRNA molecules, it has, however the disadvantage of involving multiple transfer steps into new containers.

Beside reducing background the signal to noise ratio can be improved by binding more than one label to a target

molecule or by creating many copies of an initial target molecule (amplification). For example, DNA may be detected by a method using an amplification oligonucleotide with a branched structure that can specifically bind to the target molecule and at the same time to many molecules carrying label resulting in an up to 500-fold amplification of specific signal. This method can potentially be carried out in a single tube. Its reported sensitivity is 60,000 target DNA molecules with an assay duration of 4 hours (48). Further methods of amplification include the hybridization dependant replication of RNA via the Q-β bacteriophage system (49) or polymerase chain reaction based replication of a cDNA copy of the target mRNA molecule (50). The methods described herein above, have not been used by others in the context of this invention. However, in light of the subject invention, it would be clear to those skilled in the art that use of these methods would facilitate the practice of this invention.

The invention also provides the use of a reporter gene whose product is easily detectable. The reporter gene may encode a luciferase, chloramphenicol acetyltransferase, β glucuronidase, β galactosidase, neomycin phosphotransferase, or guanine xanthine phosphoribosyltransferase.

The present invention also provides a screening method for determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene-of-interest which comprises separately contacting each of a plurality of substantially identical molecules, e.g. more than about 10^6 samples, preferably more than about

10^6 samples.

In theory, the ideal screening method provides contacting one sample with a predetermined amount of each molecule to be tested. In practice, the method may include contacting at least 3 substantially identical samples, with a predetermined amount of each molecule to be tested, more practically contacting about 9 substantially identical samples with a predetermined amount of each molecule to be tested.

Moreover, the invention provides a method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes of interest in a panel of such genes. The method comprises essentially simultaneously screening the molecules against each of the genes of interest by separately contacting each of a plurality of substantially identical samples, each of which contains a predefined number of cells, with a predetermined amount of a different molecule to be tested.

The screening method described hereinabove also provides a quantitative testing range wherein at least about 10^3 samples per week are contacted with different molecules against either one or a panel of such genes of interest.

Further, in the practice of this invention a method for determining whether a molecule not previously known to be a modulator of protein biosynthesis is capable of transcriptionally modulating the expression of a gene-of-interest is provided. This method works in human and in animal cells. Data is presented so that one skilled in the art could make the method work in fungal cells. In

addition, one skilled in the art would realize that the method may be effected in plant cells or in bacterial cells. In the case wherein luciferase is used as a reporter, this enzyme has been shown by others to be active in plant cells (89).

The invention also provides a method for transcriptionally modulating in a multicellular organism, the expression of a gene-of-interest, the expression of which is associated with a defined physiological or pathological effect in the organism. The method comprises administering, e.g. oral administration, administration as a suppository, topical contact, intravenous, intramuscular or subcutaneous administration, to the organism an amount of a molecule effective to transcriptionally modulate expression of the gene thereby affecting the defined physiological or pathological effect. The molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene-of-interest, and (c) binds to DNA or RNA or binds to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the organism. Moreover, the binding of a ligand to the ligand binding domain is normally associated with the defined physiological or pathological effect.

In the practice of the invention, examples of a multicellular organism include, but are not limited to, a human, an animal, or a plant.

The defined pathological effect may be associated with a disorder and the modulated expression of the gene-of-interest may be associated with amelioration of the disorder. Further, examples of disorders include but are

not limited to, a selection from the group consisting of cancer, a hematopoietic dysfunction, diabetes, tissue inflammation, atherosclerosis, dysfunctions of memory or learning, dysfunctions in a cholesterol or other metabolic pathway; viral, fungal or parasitic infections. Thus, the gene-of-interest is not necessarily part of the normal genetic make-up of the multicellular organism but rather can be introduced via infection by a pathogen. In one example of the invention, the defined physiological effect is growth and the organism is an animal such as a man, a cow, a pig, a bird, a fish, a sheep or a horse. In another example of the invention the defined physiological or pathological effect is an agronomically important trait. In a further example of the invention administration comprises topical contact. Further in another example of the invention, administration comprises oral, transdermal, intravenous, intramuscular or subcutaneous administration. Further in one example of the invention, provides the gene-of-interest encodes a naturally occurring receptor. Further, in one example of the invention, the receptor is a testosterone receptor. Further, in another example of the invention the receptor is an estrogen receptor. Further, in another example of the invention, the receptor which naturally occurs in the cell is a testosterone receptor. Further, in another example of the invention the receptor which naturally occurs in the cell is an estrogen receptor. Further, in another example of the invention the gene-of-interest encodes a TGF-β receptor. Further, in another example of the invention the TGF-β is TGF-β1. Further, in another example of the invention the TGF-β is TGF-β2. Further, in another example of the invention the TGF-β is TGF-β3. Further, in another example of the invention the gene-of-interest encodes an oncogene. Further, in another example of the invention the oncogene

is the neu oncogene. Further, in another example of the invention the oncogene is selected from the group consisting of H-, N-, and K-ras oncogenes.

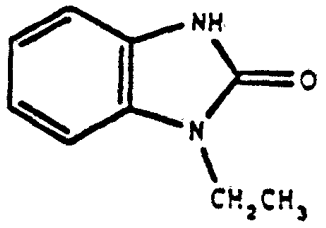
Additionally, the method for transcriptionally modulating in a multicellular organism the expression of a gene-of-interest provides that growth may be the defined physiological effect and the organism is an animal such as a cow, a pig, a bird, a fish, a sheep, or a horse.

Further, the method for transcriptionally modulating in a multicellular organism the expression of a gene-of-interest provides that the agronomically important trait may be the defined physiological or pathological effect.

Additionally, the invention provides a method for enhancing the

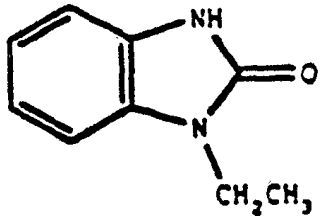
expression of human growth hormone by a

cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:



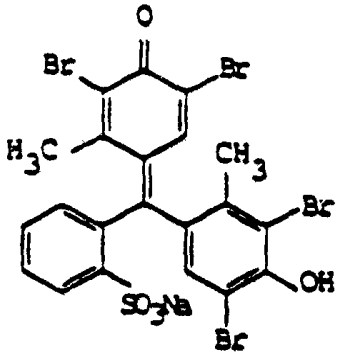
effective to enhance the expression of human growth hormone by the cell.

Additionally, this invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:



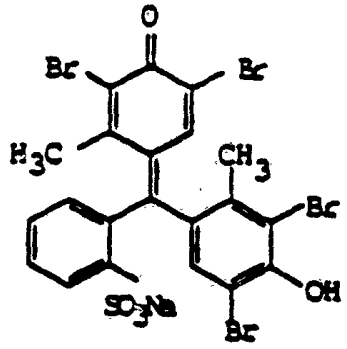
effective to enhance expression of human growth hormone by, and thus growth of, the human being.

The present invention also provides a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:



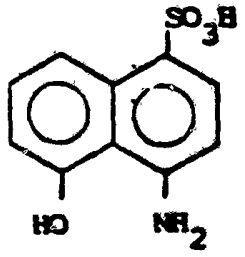
effective to enhance the expression of human growth hormone by the cell.

Another method of the invention provides increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:



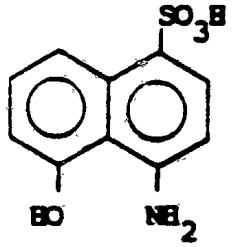
effective to enhance expression of human growth hormone by, and thus growth of, the human being.

This invention also concerns a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:



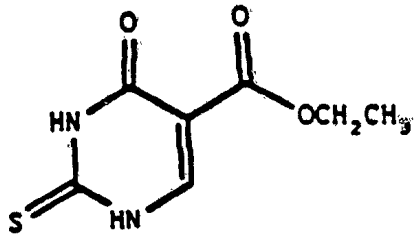
effective to enhance the expression of human growth hormone by the cell.

Further, this invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:



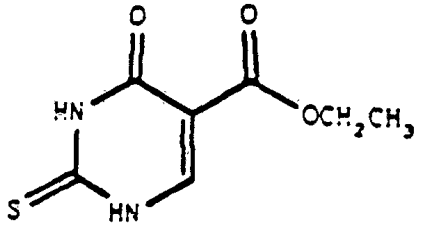
effective to enhance expression of human growth hormone by, and thus growth of, the human being.

Additionally, this invention concerns a method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:



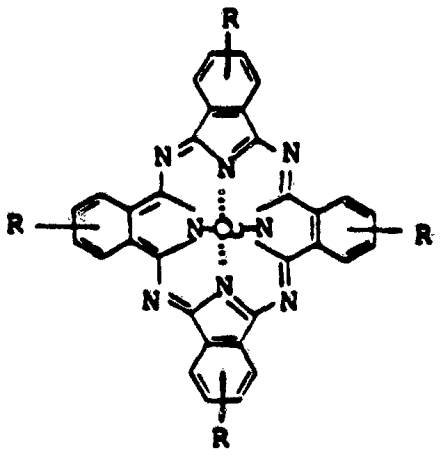
effective to enhance the expression of human growth hormone by the cell.

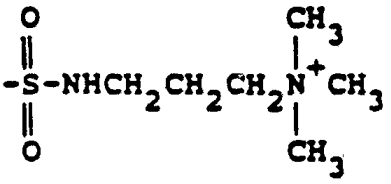
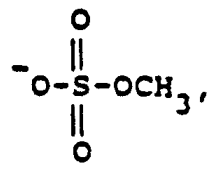
The present invention further provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:



effective to enhance expression of human growth hormone by, and thus growth of, the human being.

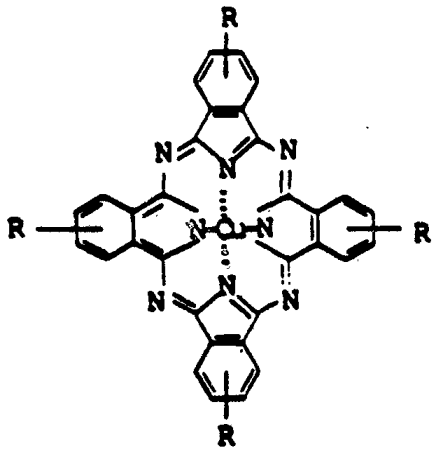
The invention provides another method for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:

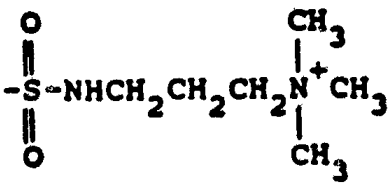
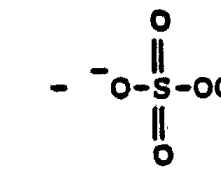


wherein R is  or ,

effective to enhance the expression of human growth hormone by the cell.

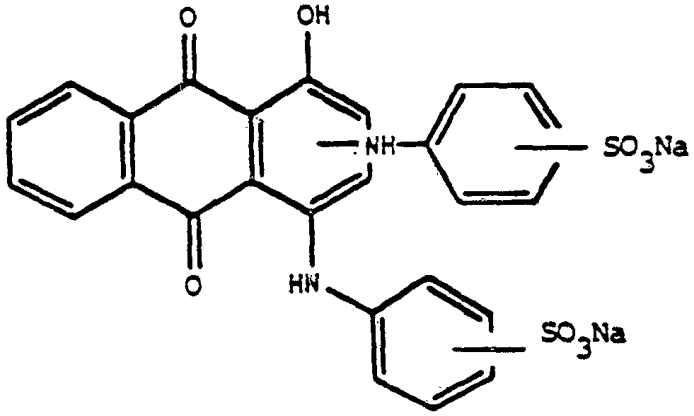
Additionally, the invention provides a method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising administering to the human being an amount of a molecule having the structure:



wherein R is  or ,

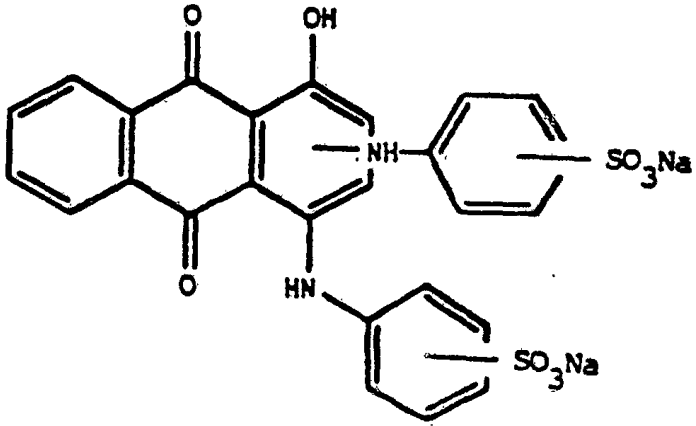
effective to enhance expression of human growth hormone by, and thus growth of, the human being.

Further, another method of the invention provides for enhancing the expression of human growth hormone by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises contacting the cell with an amount of a molecule having the structure:



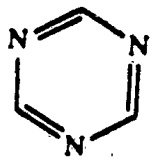
effective to enhance the expression of human growth hormone by the cell.

The invention provides yet another method of increasing the growth of a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, human growth hormone. The method comprises administering to the human being an amount of a molecule having the structure:



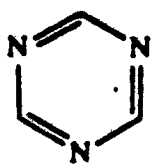
effective to enhance expression of human growth hormone by, and thus growth of, the human being.

The present invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



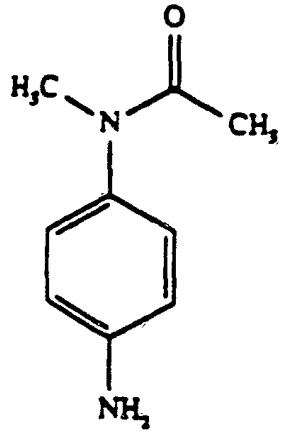
effective to enhance the expression of G-CSF by the cell.

The present invention also provides a method for increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:



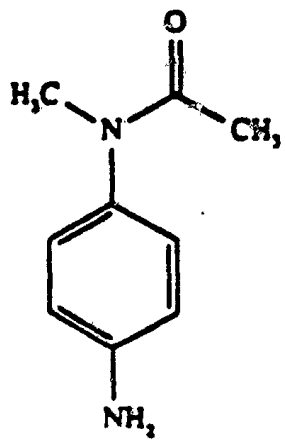
effective to enhance the expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

The present invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



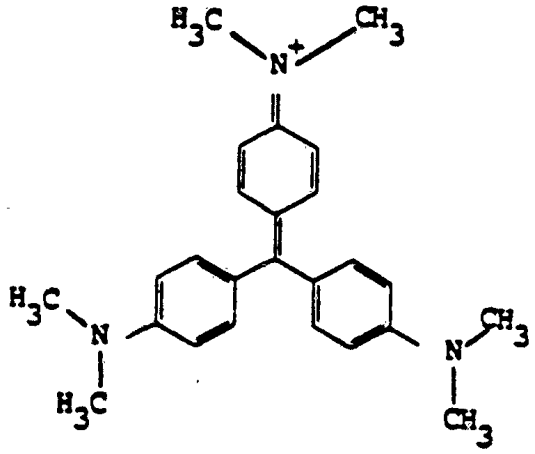
effective to enhance the expression of G-CSF by the cell.

Additionally, the invention provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:



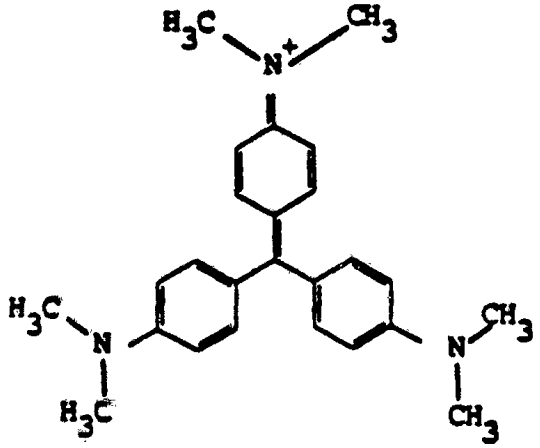
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in the human being.

A method of the invention involves enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



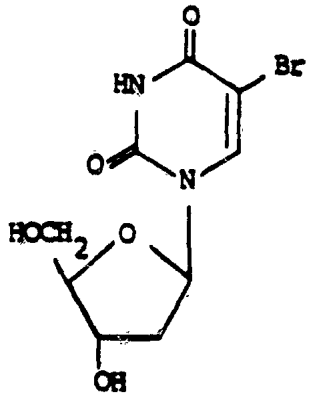
effective to enhance the expression of G-CSF by the cell.

Still another method of the invention involves increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:



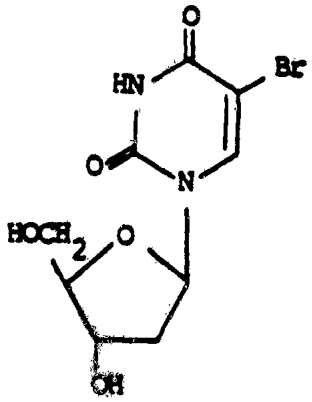
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

The present invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising contacting the cell with an amount of a molecule having the structure:



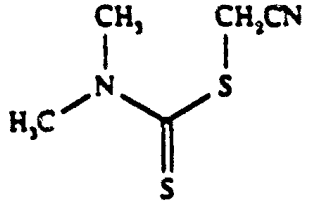
effective to enhance the expression of G-CSF by the cell.

Additionally, the invention concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



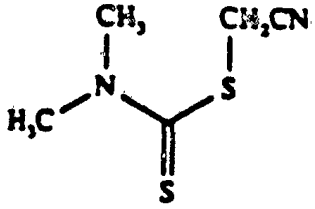
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

This invention further concerns a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



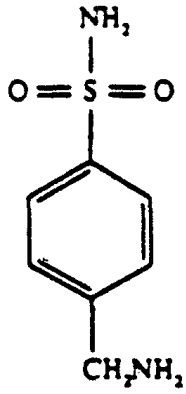
effective to enhance the expression of G-CSF by the cell.

Further provided is a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



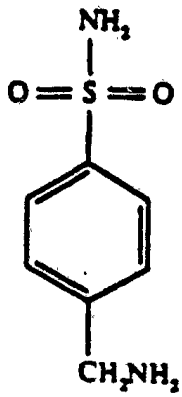
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Additionally, the present invention concerns a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising contacting the cell with an amount of a molecule having the structure:



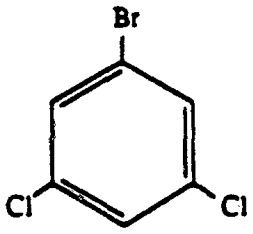
effective to enhance the expression of G-CSF by the cell.

Also, the invention provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF, wherein the method comprises administering to the human being an amount of a molecule having the structure:



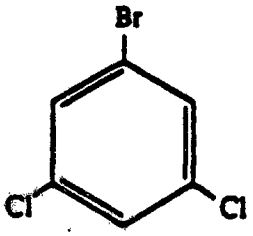
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

Further, the invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



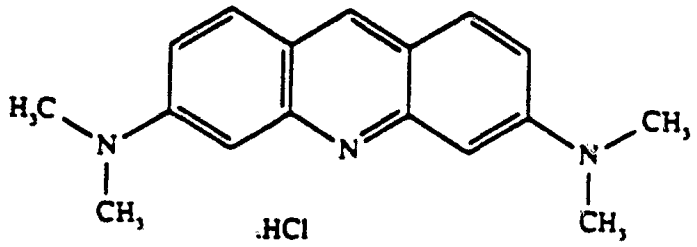
effective to enhance the expression of G-CSF by the cell.

The invention additionally concerns a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, wherein the method comprises administering to the human being an amount of a molecule having the structure:



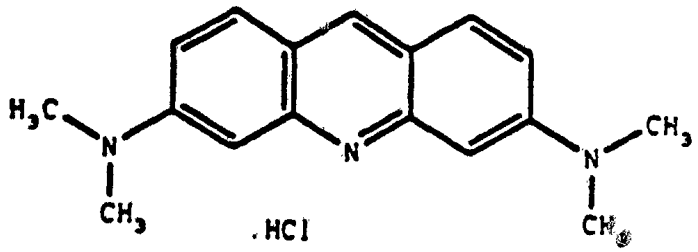
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

The invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



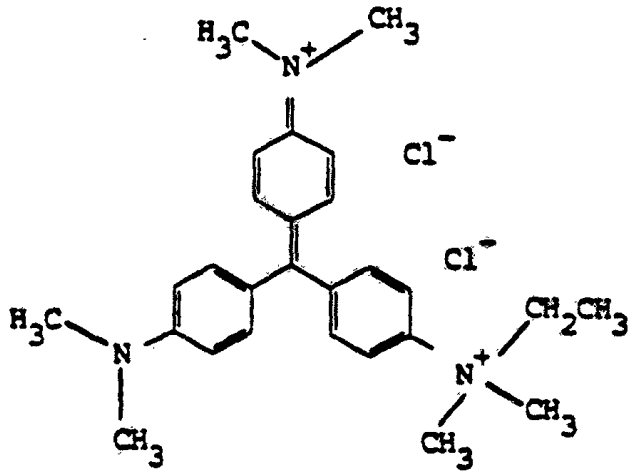
effective to enhance the expression of G-CSF by the cell.

The invention further provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:



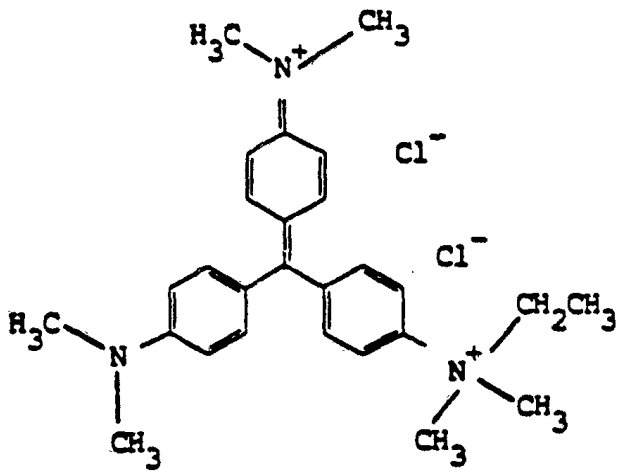
effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in the human being.

Additionally, the invention provides a method for enhancing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



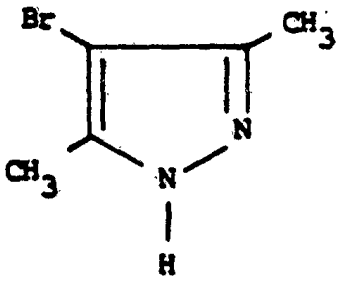
effective to enhance the expression of G-CSF by the cell.

Further, the present invention provides a method of increasing the formation of neutrophils in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing G-CSF. The method comprises administering to the human being an amount of a molecule having the structure:



effective to enhance expression of G-CSF by, and thus increase the formation of neutrophils in, the human being.

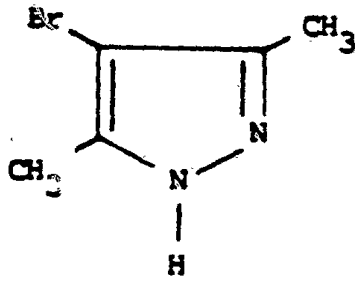
This invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the cell.

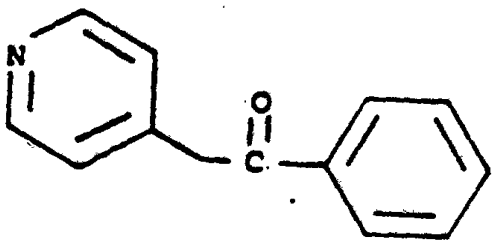
Additionally, this invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, such as

superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



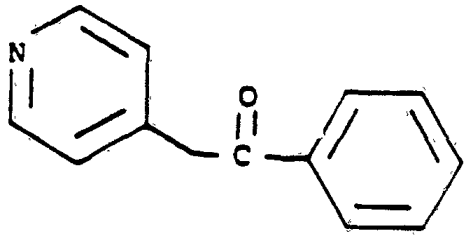
effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

Further, the invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



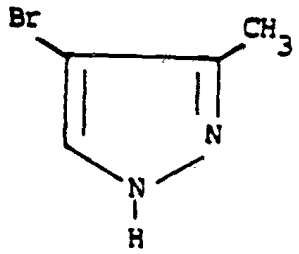
effective to decrease the expression of G-CSF by the cell.

Additionally, this invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, such as superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



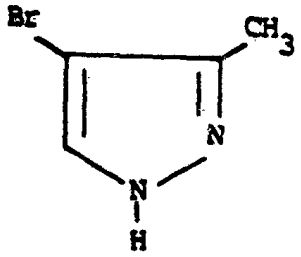
effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

The invention further provides a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the cell.

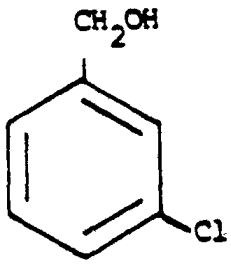
Additionally, this invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, such as superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

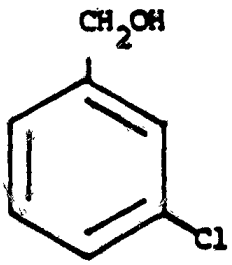
Further, the present invention a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF.

The method comprises contacting the cell with an amount of a molecule having the structure:



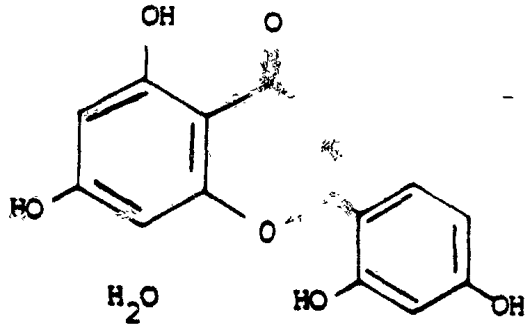
effective to decrease the expression of G-CSF by the cell.

Additionally, this invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, such as superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



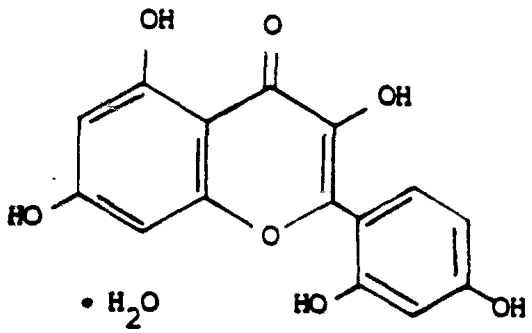
effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

The present invention further provides a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



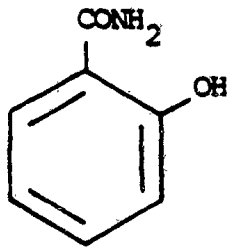
effective to decrease the expression of G-CSF by the cell.

Further still the invention provides a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, such as superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

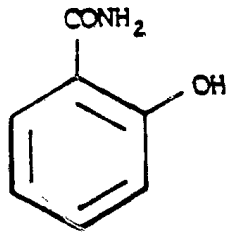
The present invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the cell.

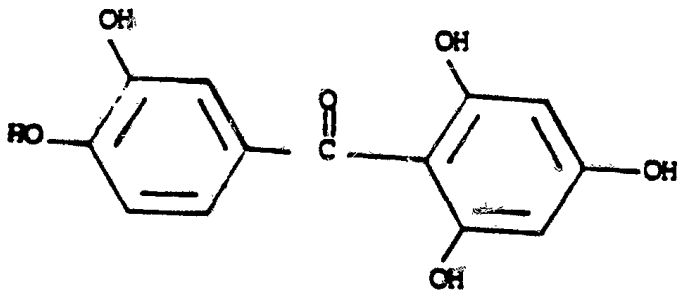
Additionally, the present invention concerns a method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils, such as superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF,

comprising administering to the human being an amount of a molecule having the structure:



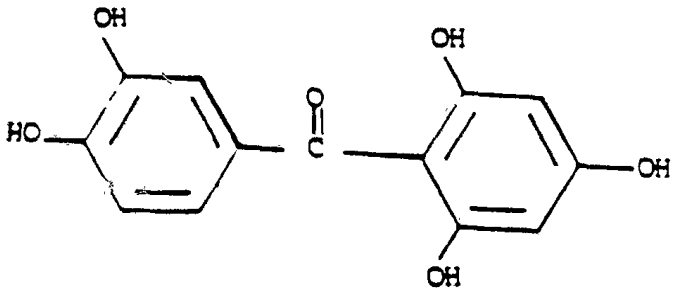
effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

The present invention also concerns a method of decreasing the expression of G-CSF by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF. The method comprises contacting the cell with an amount of a molecule having the structure:



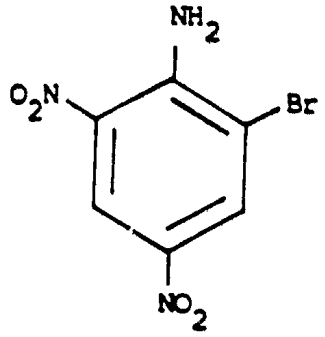
effective to decrease the expression of G-CSF by the cell.

Further, the invention concerns a method of decreasing the formation of neutrophils and effecting the metabolic functions of neutrophils, such as superoxide or peroxide formation, which has implications for the treatment of inflammatory and autoimmune disorders in a human being who (i) comprises DNA encoding, and (ii) is capable of expressing, G-CSF, comprising administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and affect the metabolic functions of neutrophils in, the human being.

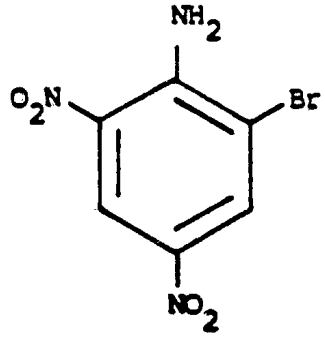
Moreover, the invention provides a method of decreasing the expression of a mammary tumor virus, such as a mouse mammary tumor virus, by a cell which (i) comprises DNA encoding, and (ii) is capable of expressing, a mammary tumor virus. The method comprises contacting the cell with an amount of a molecule having the structure:



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effective to decrease the expression of a mammary tumor virus by the cell.

Finally, the invention also provides a method of suppressing the proliferation of a mammary tumor virus in a subject who (i) comprises DNA encoding, and (ii) is capable of expressing, a mammary tumor virus, comprising administering to the subject, e.g. a human subject or an animal subject, an amount of a molecule having the structure:



effective to suppress the proliferation of a mammary tumor virus in the subject.

This invention is illustrated in the Experimental Details and Results sections which follow. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to,

limit in any way the invention as set forth in the claims which follow.

EXPERIMENTAL DETAILS

MATERIALS AND METHODS

A. Cell Culture

All media and reagents used for routine cell culture were purchased from Gibco (Grand Island, NY), Hazelton (Lenexa, KS), or Whittaker M.A. Biologicals (Walkersville, MD). Fetal calf serum (FCS) was from Hyclone (Logan, UT), and nutrients used for serum-free defined media were purchased from Sigma (St. Louis, MO), Boehringer Mannheim (Indianapolis, IN), Bachem (Torrance, CA) and Collaborative Research (Bedford, MA).

NIH/3T3 fibroblast cells (ATCC number CRL 1658) were used for transfection of plasmids containing the mouse mammary tumor virus (MMTV) promoter linked to firefly luciferase coding sequences (see below). Cells were propagated in Dulbecco's modified Eagle's medium (DMEM) obtained from Gibco, Grand Island, NY and supplemented with 10% FCS. For high-throughput (HTP) screening, transfected NIH/3T3 clones were transferred to serum free defined medium consisting of Iscove's modified Eagle's medium (IMEM) and Ham's F12 medium (1:1) supplemented with growth factors, hormones and nutrients as described previously (43).

A rat pituitary cell line, designated GC, (4, 25) was used for transfection of plasmids containing the human growth hormone promoter (see below) and was maintained in DMEM and Ham's F12 medium (1:1), supplemented with 12.5% FCS. For HTP screening, transfected GC clones were transferred to serum free defined medium consisting of DMEM and Ham's F12 medium (1:1) supplemented with growth factors, hormones and nutrients as described previously

(17, 5).

A human bladder carcinoma cell line (U5637, ATCC number HTB 9) was used for transfection of plasmids containing the human Granulocyte-Colony Stimulating Factor (G-CSF) promoter (see below) and was maintained in RPMI medium supplemented with 10% FCS. For HTP screening, transfected 5637 clones were transferred to a serum free defined medium identical to that used for the NIH/3T3 clones.

G418 (Geneticin, Gibco) at 0.2 mg/ml was routinely added to both serum and serum free defined media for maintenance of cell lines transfected with the neomycin resistance gene.

B. Plasmid Construction and Molecular Cloning of Promoter-Reporter Fusion Constructs to be Transfected into Cells Used for a 2,000-Chemical Transcription Screen

This section describes (a) the molecular cloning of the human G-CSF promoter and adjacent 5' transcriptionally modulatable regulatory sequences and (b) the making of constructs where these regulatory sequences or those of the human growth hormone (hGH) gene or those of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) control the expression of the firefly luciferase gene. These constructs were transfected into cells as described in Section C and used for a high-throughput pilot screen of 2,000 chemicals to identify chemicals acting as specific transcriptional modulators (see Section E and "Results").

Unless otherwise indicated cloning procedures were

performed essentially according to Maniatis et al. (1982) (28). Oligonucleotides were synthesized by the beta-cyanoethyl phosphoramidite method according to protocols provided by the manufacturer of the DNA-synthesizer (Model 380A, Applied Biosystems (Foster City, CA).

1. Construction of the MMTV Promoter-Luciferase fusion plasmid (pMluci)

The firefly luciferase gene was removed from the plant expression plasmid pDO432 (33) (Figure 1) as a 1.9 kb BamHI fragment and cloned into the BamHI site of pSVL (Pharmacia, Piscataway, NJ), a mammalian expression vector containing the SV40 promoter. The resulting plasmid (pSVLuci; Figure 2) was digested with XhoI and SalI to produce a 2.4 kb fragment containing the luciferase coding sequences and the SV40 late polyadenylation site. This fragment was inserted into the XhoI site of pMSG (Pharmacia, Piscataway, NJ), a eucaryotic expression vector containing the MMTV promoter. The resulting MMTV promoter-luciferase fusion plasmid (pMLuci; Figure 3) was used to transfect NIH/3T3 cells as described below (section C1). Similar constructs can be made using luciferase vectors from Clontech (Palo Alto, CA).

2. Construction of the human growth hormone (hGH) promoter-luciferase fusion plasmid

The SalI-XhoI fragment of pSVLuci (Figure 2) containing the luciferase coding sequences and the SV40 late polyadenylation site was inserted into pUC 8 (Biorad, Richmond, CA), which had been linearized by a SmaI/HinCII digestion and ligated to XhoI linkers (New England

Biolabs, Beverly, MA). The new plasmid thus generated (pUXLuci; Figure 4) was linearized by XhoI digestion followed by incubation with the Klenow fragment of E. coli DNA polymerase and the four deoxyribonucleotides to fill in the single-stranded ends of the vector. This linear (5.1 KB) form of pUXLuci was then ligated to the filled-in 550bP HindIII-XbaI fragment of the plasmid phGH:CAT (Figure 5) (25). Human growth hormone promoter sequences located on the HindIII-XbaI fragment were thus fused to the luciferase coding sequences located on pUXLuci generating the plasmid phGH-Luci (Figure 6), which was used in transfections of GC cells as described below (Section C2).

3. Construction of the human Granulocyte-Colony stimulating factor (hG-CSF) Promoter-Luciferase fusion plasmid (pG-Lucl)

Information on the G-CSF upstream and coding sequences was published by Nagata et al. (1986) and was used to synthesize 5 oligonucleotide probes (OL-1 to OL-5) to screen a human leukocyte genomic DNA library (Clontech, Palo Alto, CA) according to the supplier's instructions. The sequences of the oligonucleotide probes were:

5' GCTTTTTGTTCCAACCCCCCTGCATT 3' (OL-1);
5' CCCTGCATTGTCTTGGACACCAAAT 3' (OL-2);
5' GCGCTCCAGGAGAAGCTGGTGAGT 3' (OL-3);
5' AAGCTGATGGGTGAGTGTCTTGGC 3' (OL-4);
5' ATCAGCGGCTCAGCCTTCTT 3' (OL-5);

The sequences of OL-1, OL-2 and OL-5 recognize the G-CSF promoter region, OL-4 recognizes the first intron/exon junction and OL-3 recognizes sequences within the second exon (32). One of the clones isolated from the leukocyte

library using these oligonucleotide probes contains a 3.5 kb SalI-BamHI fragment of G-CSF genomic sequence consisting of 3.3 kb of promoter sequence and two hundred base pairs of the coding region. This fragment was inserted into the vector pGEM-7-Zf (Promega, Madison, WI) which had previously been digested with SalI/ BamHI, resulting in the vector pJM710 (Figure 7). pJM710 was then digested with PstI, and the resulting 1.6 kb fragment containing G-CSF upstream sequences and the first 15 bases of the G-CSF leader sequence was inserted into the PstI site of pGEM5-Luci (Figure 8) to generate pG-Lucl (Figure 9). This construct was then used for transfections of 5637 human bladder carcinoma cells as described below in section C3. pGEM5-Luci (Figure 8) had previously been constructed by inserting the XbaI/SalI fragment from pSVLuci (Figure 2) containing the luciferase coding sequence and the SV40 late polyadenylation signal into pGEM 5-Zf (Promega, Madison Wl) digested with XhoI/SalI.

C. Construction of Single Cell Clones Containing Various Promoter-Luciferase Fusion Constructs

1. pMluci

pMluci (Figure 3) and pSV2Neo, an antibiotic resistance plasmid (34), were co-transfected into NIH/3T3 mouse fibroblast cells using the calcium phosphate precipitation method (15) with a commercially available kit (Pharmacia, Piscataway NJ). Two days later, cells were transferred to media containing 0.4 mg/ml G418 and were grown for an additional 10-14 days. G418-resistant clones were isolated by standard methods. Once sufficient cell numbers were obtained, clones were analyzed based on several criteria: constitutive

luciferase production, induction of luciferase expression by dexamethasone (1 μM Sigma, St. Louis, MO), satisfactory attachment to microtiter plates used in the high-throughput screen (see section E) and acceptable standard deviation in multiple luciferase expression assays (see below for assay protocol). This analysis was carried out using the luciferase assay conditions described in sections D and E provided herein. Of the clones which satisfied the above criteria for the high throughput screen, one clone, M10, was selected for use.

2. phGH-LUCI

phGH-LUCI (Figure 6) and pRSVNeo, an antibiotic resistance plasmid (14), were co-transfected into GC rat pituitary cells as described above. Selection of G418-resistant cell clones was described above except for using a concentration of 0.2 mg/ml G418. Analysis of the cell clones was performed as above, except that known inducers of hGH expression (10-100 nM rat growth hormone releasing factor (rGRF, Bachem, Torrance, CA) and 10μM forskolin (Sigma, St. Louis, MO) were used in place of dexamethasone. One clone, 532, was selected for further use in the high throughput screen.

3. pG-LUC1

pG-LUC1 (Figure 9) and pRSVNeo were co-transfected into 5637 human bladder carcinoma cells as described above. Selection of G418 resistant clones was as described above except for using a concentration of 0.1 mg/ml G418. Analysis of cell clones was performed as above except that a known inducer of G-CSF expression (1-5 μg/ml lipopolysaccharide (LPS), E. coli serotype 055:b5, Difco,

Detroit, MI or Sigma, St. Louis, MO) was used in place of dexamethasone. One clone, G21, was selected for use.

D. Liquid Scintillation Counter Bioluminescence Assay

To assay for luciferase expression in transient expression assays in the various transfected clones, cells were incubated with various transcriptional inducers in serum free defined media, washed 3 times with Dulbecco's phosphate-buffered saline (D-PBS, Gibco) and lysed and measured according to the method of DeWet, et al. (100). Results were normalized to protein concentration using the Bradford protein assay (BioRad, Richmond CA) or to cell numbers using Trypan Blue (Sigma) exclusion counting in a hemocytometer (see section E).

E. High-Throughput (HTP) Screening

Cell plating: 96 well plates were treated with 50 µl per well of human fibronectin (hFN, 15 µg/ml in PBS, Collaborative Research, Bedford, MA) overnight at 37C. hFN-treated plates were washed with PBS to remove excess hFN prior to cell plating. M10, 532, and G21 cells maintained in their respective serum media (with 0.2 mg/ml G418) were washed with PBS, harvested by trypsinization, and counted using a hemocytometer and the Trypan Blue exclusion method according to protocols provided by Sigma, St. Louis, MO Chemical Company. Cells were then diluted into serum free defined media (with 0.2 mg/ml G418), and 0.2 ml of cell suspension per well was plated onto microtiter plates (532 and G21) or hFN-treated plates (M10). Plates were incubated overnight at 37C in a humidified 5% CO_2 atmosphere.

Addition of Chemicals to Cells: Chemicals from the

Oncogene Science file were dissolved in DMSO at concentrations of 3-30 mg/ml and diluted 5 fold, 110 fold, and 726 fold. 10 µl of each dilution were added to each of quadruplicate samples of cells contained in the wells of 96-well microtiter plates. Cell plates were then shaken and incubated for 6 hours at 37C, 5% CO_2.

Bioluminescence Assay: After incubation with OSI-file chemicals, cell plates were washed 3 times with PBS. Cells were lysed and bioluminescence was measured in a 96 well luminometer according to the method of DeWet, et al. (100). Data were captured using Lotus-Measure (Lotus) software and processed by custom-designed macros written in Lotus.

F. Isolation of Total Cellular RNA

Total cellular RNA was isolated from the G21 cell clone or from untransfected 5637 cells following incubation for 6 hours with various transcriptionally modulating chemicals identified in the high-throughput screen. Cells were grown in serum free medium as described above. Total cellular RNA was isolated using the RNAZol method (CINNA/BIOTECX, Friendswood, TX, Laboratories International, Inc.). Cells were resuspended and lysed with RNAZol solution (1.5 ml/9 cm petri dish) and the RNA was solubilized by passing the lysate a few times through a pipette. Chloroform was added to the homogenate (0.1 ml/1 ml), and samples were shaken for 15 seconds followed by a 5 minute incubation on ice. After centrifuging for 10 minutes, the upper phase was collected and an equal volume of isopropanol was added. Samples were incubated for 45 minutes at -20C, and the RNA was pelleted for 15 minutes at 12,000 x g at 4C. The RNA pellet was then

washed with 70% ethanol and dried briefly under vacuum.

G. Northern Blotting

Total cellular RNA was isolated from 5637 cells following incubation with chemicals as described above and electrophoresed in a 1% Agarose-Formaldehyde gel. The RNA was transferred to Duralon-UV nylon filters (Stratagene, La Jolla, CA) using the manufacturer's recommended protocol. The filters were prehybridized for 4 hours (prehybridizing solution = 5X SSC, 50 mM sodium pyrophosphate, 10X Denhardt's solution, 10% dextran sulfate, 7% SDS and 250 lg/ml denatured ssDNA) and then hybridized in the same solution for 16 hours at 65C in the presence of G-CSF or Beta-Actin (Oncor, Gaithersburg, MD) specific probes. The G-CSF probe was a 0.6kb AflII to XhoI fragment which contained most of exon 5 of the human G-CSF gene. The B-actin probe was used as a control probe to normalize for the total amount of RNA. The probes were labeled with alpha-^{32}P dCTP using a random primed DNA labeling kit (Amersham, Arlington, IL). Following hybridization, filters were first probed with, G-CSF-and Fusion reprobed with B-Actin Probe were washed three times at room temperature with 1X SSC, 0.13% SDS and three times at 65C with 0.2X SSC, 0.1% SDS. Filters were first probed with G-CSF-and then reprobed with B-actin-probe. Exposure to x-ray films was performed overnight. Bands were excised and counted in a liquid scintillation counter (LKB, Gaithersburg, MD), and counts obtained with the G-CSF specific probe were normalized relative to the counts obtained with the B-Actin specific probe.

H. S1 Nuclease protection

S1 Nuclease protection assays were carried out as described in reference 94.

I. MTT Cell toxicity assay

To determine cytotoxic concentrations of chemicals registering as positives in the High-Throughput luciferase assay the MTT cytotoxicity assay was employed (95). In this assay, a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, MTT] is reduced to a colored formazan product by reducing enzymes present only in living metabolically active cells.

J. Two-antibody Sandwich Immunoassay

Supernatants from 5637 bladder carcinoma cells incubated with chemicals registering as positives in the G-CSF promoter/luciferase High-Throughput assay were assayed for secreted G-CSF protein using the two-antibody sandwich immunoassay (96). The G-CSF Assay kit manufactured by Oncogene Science, Inc. was used and the manufacturer's instructions were followed.

K. Construction of a Yeast Expression Vector

Plasmid pHZ18 (97,98) contains 2μ DNA for propagation in S. cerevisiae, the yeast promoter cyc 1, which is compatible with expression in yeast cells, and the URA 3 gene for selection. The plasmid was linearized with BamH1, the ends were filled-in using deoxynucleotides and E.coli DNA polymerase Klenow fragment, and then the plasmid was digested with Aat II. A 4.1 kb fragment containing the cyc1 promoter, URA3 and 2μ genes was

separated by agarose gel electrophoresis and subsequently purified by electroelution onto ion-exchange paper (Whatman, DE81). Plasmid pBR322 was treated with endonucleases Aat II and Pvu II and a 2.2 kb fragment containing the plasmid's origin of replication and the amp^R gene was isolated by agarose gel electrophoresis and eluted onto DE81 paper.

The 2.2 kb pBR322 and 4.1 kb pHZ18 fragments were ligated using T4 DNA ligase according to standard procedures (94). The resulting 6.3 kb vector pHZBR was digested with BamH1 for subsequent insertion of the luciferase coding sequence downstream of the cyc1 promoter.

An Nco I - Sal I restriction fragment containing the luciferase gene starting at the second ATG, was made blunt-ended by filling in and ligated into the filled-in BamH1 site of pHZBR as readily understood by one skilled in the art. Clones of the correct orientation were identified via restriction mapping to yield plasmid pHZluci24. This plasmid was used to transform S.cerevisiae strain DB745.

L. Transformation of Yeast Cells

S.cerevisiae DB745 were made competent according to published methods (99). One and 4μg of either pHZluci24 or pHZ18 (transfection control) were added to the competent cells and incubated at 30°C for 30 minutes. Lithium acetate-PEG was mixed gently with the cells and allowed to incubate for another 45 minutes at which time the cells were shifted to 42°C for 5 minutes. The cells were spread onto uracil(-) plates and incubated at 30°C for several days. Cell colonies were picked, grown to saturation in YPD media and analyzed for luciferase

activity (99) Cell lysis and analysis of luciferase activity can be carried out by methods known to one skilled in the art (99, 100). Stock cultures were made from clones expressing luciferase positive clones.

RESULTS

A. Validation of Cell Lines

Prior to initiation of drug screening, it was demonstrated that the transfected promoter-luciferase fusion plasmids were reacting to transcriptional inducers in a manner as predicted based on the published literature. As shown in Figure 10, all three transfected cell clones chosen responded to inducers which have been reported to stimulate the endogenous genes; the MMTV-luciferase containing clone M10 was stimulated 11.6 fold by 1μM dexamethasone, the hGH-luciferase containing clone 532, was stimulated 2.2 fold by 100 nM rat growth hormone releasing factor (GRF), and the hG-CSF containing clone G21 was stimulated 5.7 fold by 5 μg/ml lipopolysaccharide (LPS).

It was also demonstrated that certain steroidal chemicals other than dexamethasone modulated luciferase expression in the cell clone M10, which harbors the MMTV promoter-luciferase fusion construct. As shown in Figure 11, dexamethasone stimulated the MMTV promoter in cell clone M10 (mouse fibroblast origin), while progesterone did not. It has been shown that a rat fibroblast cell line which contains high levels of glucocorticoid receptor but low levels of progesterone receptor, shows stimulation of the MMTV promoter by the glucocorticoid dexamethasone but not by progesterone (7). In addition,

Figure 11 shows that the mineralocorcicoid aldosterone stimulates clone M10, as is expected based on previously published work which indicates that aldosterone can act through the glucocorticoid receptor to stimulate the MMTV promoter (6).

A 7-hour incubation of G 21 cells with PMA in serum-free media increased luciferase expression directed by the G-CSF promoter by 34.6 fold in the absence and by 24.6 fold in the presence of EGF. TNF-Alpha induction did not significantly change on EGF addition (2.8 fold with and 2.1 fold without EGF).

B. High-Throughput Drug Screen

Table 1 shows a summary of the results of a one-week, high-throughput screen of 2,000 chemicals to identify those chemicals specifically stimulating or inhibiting transcription from the G-CSF, hGH or MMTV promoters. This screen concurrently tested chemicals at three concentrations on quadruplicate samples of the M10, 532 and G21 cell lines. A minimum stimulation of one promoter, to the degree indicated, and less than 50% activation of the other two promoters was required for

TABLE 1

SUMMARY OF HIGH-THROUGHPUT SCREEN

Number (%) of Chemicals Which Activate Expression:

Promoter	2-3 Fold	3-5 Fold	5-7 Fold	7-10 Fold	>10 Fold	Total
G-CSF	NA	23 (1.1%)	10 (0.5%)	3 (0.15%)	2 (0.10%)	38 (1.9%)
hGH	NA	NA	12 (0.6%)	5 (0.03%)	6 (0.03%)	23 (1.14%)
MMTV	15 (0.7%)	1 (0.05%)	0 (0%)	1 (0.05%)	1 (0.05%)	18 (0.9%)

Number (%) of Chemicals Which Inhibit Expression >3 Fold

Promoter		
G-CSF	7	(0.35%)
hGH	42	(2.1 %)
MMTV	1	(0.05%)

TABLE 2

A) TRANSCRIPTIONAL ACTIVATORS

Chemical #	Chemical Name	FOLD INDUCTION RELATIVE TO SOLVENT CONTROL GCSF	hGH	MMTV
G-CSF:				
40	3-Acetyl-2-6-Bis(tertiary butyl amino)-4-methyl-pyridine	5.62	0.62	0.27
58	1-Acetylimidazole	6.03	0.17	0.42
237	N-Carbethoxy-phthalimide	4.77	0.06	0.62
254	1-(2-Chloroethyl)piperidine	4.09	0.90	0.98
364	Melamine	3.67	1.18	1.07
473	1,3,5,-Triazine	>3	0.50	0.87
542	5-Bromo-2'-deoxycytidine	6.28	1.08	1.26
543	5-Bromo-2'-deoxyuridine	7.17	0.72	0.98
878	Blueberry leaf extract	3.84	1.17	0.78
1025	Culvers Root extract	4.09	0.98	1.24
1234	4-Aminocinnamic Acid hydrochloride	4.87	0.51	1.03
1255	1-Bromo-3,5-dichlorobenzene	5.74	0.43	1.09
1374	4'-Amino-N-methylacetanilide	11.03	0.05	1.05
1375	4'-(aminomethyl)benzene sulfonamide hydrochloride	8.94	0.04	1.37
1376	2-Amino-5-Methyl benzene sulfonic acid	6.37	0.04	1.32
1397	5-Amino-3-methylisothiazole hydrochloride	3.63	0.57	1.13
1482	2-Aminophenyl disulfide	3.99	0.54	1.07
1483	4-Aminophenyl disulfide	4.64	0.38	1.09

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
1521	2-Amino-6-purinethiol	3.59	0.73	0.92
1583	8-Bromoadenosine	5.82	0.12	0.88
1592	Bis(2,2,3,3,4,4,5,5,6,6,7,7,) dodecafluoroheptyl-(+)-camphorate	3.20	0.74	1.34
1783	Cupferron	6.55	0.32	0.89
1793	Cyanomethyl-N,N-dimethyl dithiocarbamate	9.50	0.52	1.21
1994	3-Bromobiphenyl	3.29	0.34	0.63
2001	1-Bromo-4-tertiary butyl benzene	3.11	0.74	1.12
2030	4-Bromo-2-fluoro-6-nitroanizol	5.53	0.67	0.87
2096	(+)-1-Bromo-3-Chloro-2methyl propane	3.27	0.61	0.89
2097	1-Bromo-5-Chloro pentane	5.09	0.88	1.22
2129	4-Chlorobenzyl Chloride	3.23	0.75	0.95
GROUP A:				
378	7-Oxo-7H-benzo[e]perimidine 4-carboxylic acid	4.12	0.26	0.59
423	Quinacrine dihydrochloride hydrate	2.39	0.56	0.64
427	Resazurin	3.14	0.43	0.71
836	Thionin	3.20	0.23	0.58
1776	Cresyl Violet Acetate	3.50	0.15	1.36
1904	9-Aminoacridine hydrochloride	4.12	0.54	0.82
GROUP B:				
570	Methyl Green	>3	0.52	0.79
1780	Crystal Violet	20.39	0.38	1.15

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
GROUP A AND B:				
80	Acridine Orange	5.87	0.66	0.83
hGH:				
70	2-Acetylpyrrole	0.43	9.26	0.85
299	10,11-Dihydrocarbamazepine	0.53	5.46	0.47
322	1-ethyl-2-benzimidazolinone	0.60	11.18	1.12
325	Fisetin	0.14	5.42	1.0
552	3-(4-chlorophenyl)-1-methoxy-1-methyl urea	0.81	5.31	0.86
790	Rivanol	0.01	5.94	0.58
792	Rose Bengal	0.94	5.31	1.21
856	Tripalmitin	0.28	6.49	0.42
1004	Arnica 4x	0.85	6.48	1.22
1160	Rochester # 8180	0.38	5.79	0.80
1251	Bromocresol Green	0.14	15.19	0.33
1337	4-Amino-5-hydroxy-1-naphthalene sulfonic acid	0.07	15.87	0.23
1489	2-Amino-4-phenylthiazole hydrobromide monohydrate	0.24	5.55	0.61
1550	2-Aminothiazole	0.04	5.44	0.87
1552	2-amino-2-thiazoline	1.23	7.26	0.52
1561	4-Amino-3,5,6-trichloropicolinic acid	0.23	8.05	0.48
1598	N,N'-Bis-[3-(4,5-dihydro-1H-imidizol-2-yl)phenyl] urea dipropenoate	0.72	5.32	1.27

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
1678	4,8-Bis(hydroxymethyl)-tricyclo [5,2,1,$0^{2.6}$]decane	0.36	7.08	0.89
1740	5-carbethoxy-2-thiouracil	0.74	17.77	0.87
1747	N_6-carbobenzyloxy-L-lysine	0.78	6.16	0.86
1804	Cyclobutane carboxylic acid	1.05	9.41	0.49
1876	Alec Blue	0.87	11.91	0.40
1881	Alizarin Blue Black B	0.21	18.87	0.69
MMTV:				
189	Bathocuproinedisulfonic Acid disodium salt hydrate	1.06	1.47	2.80
453	2,2':6',2"-Terpyridine	0.79	0.58	13.30
519	b-Apo-8'-carotenal	1.15	0.68	2.76
562	Copaiva Balsam	1.10	0.15	2.34
629	Homoveratric acid	0.85	1.05	2.48
633	5-Iodorotic acid	1.02	0.86	2.46
765	Prednisolone-21-Acetate	0.96	1.30	2.66
828	2,4,5,4'-Tetrachlorodiphenylsulfide	1.47	1.34	2.20
848	Triamcinolone acetonide	0.75	1.28	2.43
944	Peanut	1.15	0.91	2.10
1269	5-Amino-4,6-dichloropyrimidine	0.72	0.91	2.18
1316	2-Aminofluorene	0.74	1.39	2.33
1318	2-Amino-9-fluorenone	1.13	0.85	2.41
1384	2-Amino-4'-methylbenzophenone	1.33	0.50	2.43

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INDUCTION GCSF	hGH	MMTV
1573	5-Bromoacenapthene	1.49	0.34	4.30
2064	4-(Bromomethyl)-6,7-dimethoxy-coumarin	0.82	1.10	2.53
2148	2-chlorocyclohexanone	0.45	0.92	2.82
2191	Chloramphenicol	0.37	0.35	7.32

B) TRANSCRIPTIONAL INHIBITORS

Chemical#	Chemical Name	FOLD INHIBITION RELATIVE TO SOLVENT CONTROL GCSF	hGH	MMTV
G-CSF:				
209	4-Benzoylpyridine	6.66	1.08	0.81
371	Morin hydrate	11.11	0.41	0.89
660	Maclurin	10.0	0.34	1.04
798	Salicylamide	4.76	0.90	0.68
2009	4-Bromo-3,5-dimethylpyrazole	3.70	0.57	0.64
2082	4-Bromo-3-Methylpyrazole	5.26	0.65	1.23
2121	3-Chlorobenzyl alcohol	4.76	0.40	1.14
hGH:				
183	Auramine O	0.72	4.00	0.70
240	Carminic acid	0.63	5.26	0.80
443	Sulfamethazine	0.60	4.76	0.79
512	Amaranth	0.81	5.26	0.68

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INHIBITION GCSF	hGH	MMTV
541	5-Bromo-4-Chloro-3-indoxyl-phosphate K-salt	0.90	6.25	0.86
556	Chromazurol S	0.73	33.33	0.87
561	Clove Oil	0.62	5.00	0.05
577	Na-Ne-Diacetyl-L-lysine	0.64	4.00	0.68
578	Dibenzoyl-D-tartaric acid	0.65	4.00	0.91
630	Hydantoin-5-acetic acid	0.70	3.57	0.74
640	Kernechtrot	0.64	5.00	0.59
759	Piperidine	0.64	5.88	0.95
764	Prednisolone	0.82	4.54	0.59
875	Black Walnut extract	0.69	6.25	0.80
892	Colts Foot Leaves extract	0.68	11.11	0.87
893	Comfrey Leaf extract	0.74	11.11	0.90
920	Horehound Herb extract	0.56	3.84	0.84
921	Horsetail Grass extract	0.72	3.44	0.86
942	Pau D'Arco extract	0.80	6.25	0.63
970	Thyme extract	0.57	4.34	1.07
1591	1,2-Bis(di-p-tolylphosphino)-ethane	0.56	5.55	0.96
1604	2,4-Bis[5,6-bis(4-sulfophenyl)-1,2,4-Triazine-3-yl)-pyridine, tetrasodium salt hydrate	0.77	5.00	0.97
1635	[(15)-endo]-(-)-Borneol	0.71	9.09	0.99
1640	1,2-Bis(2-pyridyl)-ethylene	0.79	5.00	0.59
1641	2,3-Bis(2-pyridyl)-pyrazine	0.83	5.55	0.60

TABLE 2 (CONT.)

Chemical#	Chemical Name	FOLD INHIBITION GCSF	hGH	MMTV
1648	2-[5,6-Bis(4-sulfophenyl)-1,2,4-triazine-3-yl]-4-(4-sulfophenyl)-pyridine, trisodium salt	0.86	7.69	1.00
1651	Bis(2,2,2-trifluoroethyl) (methocarbonyl-methyl)-phosphonate	0.69	3.57	0.70
1655	2,5-Bis(trifluoro-methyl)benzoic acid	0.54	4.76	0.81
1703	3-Bromobenzonitrile	0.76	10.00	0.90
1704	4-Bromobenzonitrile	0.77	4.16	0.94
1705	4-Bromobenzophenone	0.54	14.28	0.62
1712	Calcein Blue	0.74	8.33	0.94
1720	(1S)-(-)-Camphor	0.65	4.76	0.66
1764	7-(Carboxymethoxy)-4-Methylcoumarin	0.55	7.14	0.82
1770	Carminic acid	0.54	10.00	0.57
1771	L-Carnosine	0.71	10.00	0.72
1773	O-Cresolphthalein Complexone	0.62	10.00	0.67
1890	Alloxazine	0.80	5.26	0.58
2035	5-Bromofuroic acid	0.57	7.14	0.89
2036	8-Bromoguanosine	0.58	4.34	0.81
2037	1-Bromohexadecane	0.51	4.00	0.50
MMTV:				
2010	2-Bromo-4,6-dinitroaniline	0.80	0.63	3.57

a chemical to be considered a selective activator. A minimum inhibition of 3 fold of one promoter and less than 20% inhibition of the other two promoters was required for a chemical to be considered a selective inhibitor. Table 2 gives the names and induction or inhibition ratios of the lead chemicals identified for each promoter. Figure 12 illustrates the transcriptional stimulation and Figure 13 the transcriptional inhibition observed with some of the lead chemicals. Some of the chemicals activating G-CSF transcription fell into conspicuous groups of analogs (Table 2; Group A and B). Although not specifically indicated in Table 2, groups of homologs and analogs can also be found for G-CSF-inhibiting as well as hGH-activating chemicals.

To determine the number of lead chemicals, which reproducibly score as positives in repeated luciferase assays, two types of experiments were conducted:

1) G-CSF lead chemicals #1780, #58, #1783, #1374 were subjected to 48 independent luciferase assays performed on the same day. Compounds #58, #1780 and #1374 scored as positives in every single one of these assays inducing luciferase expression between 2 and 28-fold (#58), 20 and 80-fold (#1780) and 5 and 40-fold (#1374). Compound #1783 scored as positive only in half of the 48 repeat assays probably due to its relatively low induction of luciferase expression (1.5 to 8-fold).

2) All of the 18 lead chemicals inducing luciferase expression from the MMTV promoter were again subjected to luciferase assays: 10 chemicals (#453, #519, #562, #765, #828, #848, #1269, #1316, #1384 and #2148) again induced luciferase expression between 2.1 and 2.8-fold. Probably due to the relatively low induction level close to the

background of the assay, the other eight lead chemicals did not repeat on that particular day. The most prominent lead chemical, #453 (13.3-fold induction in the original high-throughput assay), was repeated in a total of 3 independent assays and consistently induced luciferase expression from the MMTV promoter between 10 and 35-fold. Replacing DMSO by methanol to dissolve the chemical did not affect its ability to activate the MMTV promoter.

Aqueous clarified supernatants derived from individual Actinomyces colonies prepared by standard methods known to those skilled in the art as well as corresponding methanol extracts were assayed for modulation of luciferase expression from three target promoters of interest.

Out of 356 samples tested for modulation of the three different promoters, 25 samples scored as positives, 7 of which were promoter-specific. A summary of the obtained results is contained in Figures 52 and 53. Thus high-throughput screening of fermentation broth samples using a luciferase expression assay consistently leads to the discovery of lead samples with the potential to be developed into novel pharmaceuticals.

C. Effects of Lead Chemicals on Endogenous G-CSF mRNA Levels

Northern blot analysis was used to demonstrate the stimulatory effects of lead chemicals #670 and #1255 on endogenous G-CSF mRNA levels. As shown in Figure 14, both OSI #670 and #1255 stimulated production of G-CSF mRNA, as shown by a G-CSF-specific probe, but not of actin mRNA, as shown by a b-actin-specific probe. Also shown are the effects of the solvent, DMSO, used to

dissolve the chemicals and a proteinaceous positive regulator, interferon-gamma. From these data it is concluded that chemicals, which induce luciferase expression from specific promoters, in plasmids stably integrated into cells, are also capable of stimulating mRNA production from the corresponding endogenous promoters without using a reporter system.

To further confirm, that compounds that had been identified in a luciferase expression assay using a G-CSF specific reporter cell line would be active in inducing transcription of the endogenous G-CSF gene, cells from the parental cell line 5637 used to construct the reporter cell line were incubated with cycloheximide (25 μg/ml), DMSO (0.5%,solvent control) and low, medium and high concentrations of the compounds 542 (10, 50, 250 μM),1255 (20, 100, 500 μM), 1793(0.25, 1.2, 6.25 μM) and 1904 (20, 100 μM) for 18 hours. RNA was extracted and the concentration of G-CSF, GM-CSF and gamma-actin mRNA was determined by the S1 protection method as described in Materials and Methods. The positions of G-CSF GM-CSF and gamma-actin specific protected fragments are indicated (G, GM, A) at the left side of the gel (Figure 15).

Interestingly, all four compounds tested increased the amount of G-CSF mRNA. It is of great interest that at least two of them, namely #542 and #1793, also increased the amount of GM-CSF mRNA. Compound #543, a structural analog of #542 showed similar activity.

D. Dose response Analysis of Structurally Related Lead Chemicals

Among the chemicals which specifically activated the

G-CSF promoter were groups of structural homologs. Three such homologs, #80, #670, and #1780, belong to groups listed in Table 2. These three structurally related chemicals all specifically activated the G-CSF promoter. Dose response graphs obtained with chemicals #80, #670, and #1780 are shown in Figure 16. Although these chemicals all demonstrate large maximal stimulations, it is clear that their potencies, as measured by their ED_{50}'s (concentration of chemical resulting in 50% maximal stimulation), show wide variability (5-70 μM)).

E. Effects of Lead Chemicals on Target Protein Secretion

Two of the most promising lead chemicals (# 542 and #1780), which were shown to stimulate levels of endogenous G-CSF mRNA as well as luciferase expression from the G-CSF promoter/luciferase fusion constructs, were further investigated for their ability to increase G-CSF secretion into the media of 5637 bladder carcinoma cells incubated with the chemicals for 48 hours. The levels of G-CSF in the cell supernatants were determined by a sandwich-antibody assay as described in Materials and Methods (Figure 17).

F. Cytotoxicity of Lead Chemical #542

To address the question whether the induction of G-CSF, and GM-CSF transcription by the compound #542 was a specific effect or rather a phenomenon linked to a potential sensitivity of these promoters to stress exerted by toxic compounds, the concentration dependency of induction of luciferase activity in the reporter cell line G21 was compared to the concentration dependency of inhibition of respiration in FRE cells.

Cells were seeded into 96 well microtiterplates (20,000 cells/well) and cultured overnight. Compound #542 was added at various concentrations and the cells were incubated for 6 hours. The MTT-colorimetric assay was carried out on identically treated samples of FRE cells. Induction of luciferase reporter signal (plain line) and on inhibition of respiration (dashed line) are plotted versus the concentrations of compound (Figure 18). The ED50 for induction of luciferase activity differed from the ED50 for inhibition of respiration by a factor of almost 10, which might indicate that compound #542 exerts a specific effect on G- and GM-CSF transcription.

G. Luciferase expression assay in yeast

To determine, whether the luciferase expression assay would also be useful to study other than mammalian cells, a yeast expression plasmid carrying the luciferase gene under control of a yeast promoter was constructed and transfected into appropriate yeast cells. Luciferase activity was demonstrated using the procedures described in Materials and Methods and stock cultures were made from position clones.

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The claims defining the invention are as follows:

1. A method of transcriptionally modulating the expression of a gene of interest which encodes a protein, the expression of which is associated with a defined physiological or pathological effect within a multicellular organism, which includes contacting a eukaryotic cell, which is capable of expressing the gene, with a molecule at a concentration effective to specifically transcriptionally modulate expression of the gene and thereby affect the level of the protein encoded by the gene of interest which is expressed by the cell, which molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene of interest, and (c) binds to DNA or RNA, binds to a protein through a domain of such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological effect.

2. A method of claim 1, wherein the molecule does not naturally occur in any cell of a higher eucaryotic organism.

3. A method of claim 1, wherein the molecule does not naturally occur in any cell.

4. A method of claim 1, wherein the molecule is not a naturally occurring molecule.

5. A method of claim 1, wherein the cell is a cell of

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the multicellular organism.

6. A method of claim 1, wherein the cell is an animal cell.

7. A method of claim 6, wherein the animal cell is a human cell.

8. A method of claim 1, wherein the cell is a plant cell.

9. A method of claim 1, wherein the cell is a fungal cell.

10. A method of claim 1, wherein the cell is a protozoan cell.

11. A method of claim 1, wherein the cell is a bacterial cell.

12. A method of claim 1, wherein the gene-of-interest is a human gene.

13. A method of claim 1, wherein the gene-of-interest encodes a hematopoietic protein.

14. A method of claim 13, wherein the hematopoietic protein is a colony stimulating factor.

15. A method of claim 14, wherein the colony stimulating factor is granulocyte-macrophage colony stimulating factor (GM-CSF).

16. A method of claim 14, wherein the colony

stimulating factor is granulocyte colony stimulating factor (G-CSF).

17. A method of claim 14, wherein the colony stimulating factor is macrophage colony stimulating factor (M-CSF).

18. A method of claim 13, wherein the hematopoietic protein is erythropoietin (EPO).

19. A method of claim 1, wherein the gene-of-interest encodes an interleukin (IL).

20. A method of claim 1, wherein the gene-of-interest encodes a growth hormone selected from the group consisting of human, bovine, porcine, avian, ovine, piscine, and equine growth hormone, and polypeptide analogs thereof having the biological activity of the corresponding naturally occurring growth hormone.

21. A method of claim 1, wherein the gene of interest is a growth hormone releasing factor.

22. A method of claim 1, wherein the gene-of-interest is a viral gene.

23. A method of claim 22, wherein the viral gene is a retroviral gene.

24. A method of claim 23, wherein the retroviral gene is a gene from the HIV, HTLV-1, or HTLV-2 virus.

25. A method of claim 22, wherein the viral gene is a

gene from a hepatitis virus.

26. A method of claim 22, wherein the viral gene is a gene from a herpes virus.

27. A method of claim 22, wherein the viral gene is a gene from an animal virus.

28. A method of claim 22, wherein the viral gene is a gene from a papilloma virus.

29. A method of claim 22, wherein the viral gene is a gene from a cytomegalovirus.

30. A method of claim 27, wherein the animal virus is a pseudorabies, Marek's, Newcastle's Disease, or IBR virus.

31. A method of claim 1, wherein the gene-of-interest is a plant gene.

32. A method of claim 31, wherein the plant gene encodes an agronomically important trait.

33. A method of claim 32, wherein the agronomically important trait is selected from the group consisting of germination, sprouting, flowering, fruit ripening, salt tolerance, herbicide resistance, pesticide resistance, fungicide resistance, temperature resistance, and growth.

34. A method of claim 1, wherein the gene-of-interest is a protozoan gene.

35. A method of claim 34, wherein the protozoan is

selected from the group consisting of Trypanosoma, Plasmodium, Leishmania, Giardia, Entamoeba, Toxoplasma, Babesia, and Cryptosporidiosis.

36. A method of claim 1, wherein the gene-of-interest is a helminth gene.

37. A method of claim 1, wherein the gene-of-interest is an oncogene.

38. A method of claim 37, wherein the oncogene is the phl-abl oncogene.

39. A method of claim 37, wherein the oncogene is selected from the group consisting of H-, N-, and K-ras oncogenes.

40. A method of claim 37, wherein the oncogene is the neu oncogene.

41. A method of claim 37, wherein the oncogene is the src oncogene.

42. A method of claim 1, wherein the gene-of-interest encodes TGF-β1.

43. A method of claim 1, wherein the gene-of-interest encodes TGF-β2.

44. A method of claim 1, wherein the gene-of-interest encodes TGF-β3.

45. A method of claim 1, wherein the gene-of-interest encodes a naturally occurring receptor.

46. A method of claim 45 wherein the receptor is a receptor for a TGF-β.

47. A method of claim 45, wherein the receptor is a testosterone receptor.

48. A method of claim 45, wherein the receptor is an estrogen receptor.

49. A method of claim 45, wherein the receptor is the human low density lipoprotein (LDL) receptor.

50. A method of claim 45, wherein the receptor is the receptor for a hematopoietic protein selected from the group consisting of M-CSF, G-CSF, GM-CSF, and EPO.

51. A method of claim 45, wherein the receptor is the receptor for an interleukin (IL) selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 and IL-8.

52. A method of claim 45, wherein the receptor is a cell surface protein which mediates infection of the cell by a virus.

53. A method of claim 52, wherein the virus is selected from the group consisting of HIV, HTLV-1, HTLV-2, a hepatitis virus, a herpes virus, an animal virus, a papilloma virus, a cytomegalovirus, and a rhinovirus.

54. A method of claim 1, wherein the receptor which naturally occurs in the cell is a testosterone receptor.

55. A method of claim 1, wherein the receptor which naturally occurs in the cell is an estrogen receptor.

56. A method of claim 1, wherein in (c) the protein is not the protein encoded by the gene-of-interest.

57. A method of determining whether a chemical not previously known to be a modulator of protein biosynthesis is capable of specifically transcriptionally modulating the expression of a gene-of-interest which includes:

(a) contacting a sample which contains a predefined number of identical eucaryotic reporter cells with a predetermined concentration of the chemical to be tested, each such reported cell being a transfected parental cell including a single DNA construct consisting essentially of in 5' to 3' order (i) a modulatable transcriptional regulatory sequence of the gene-of-interest, (ii) a promoter of the gene-of-interest, and (iii) a reporter gene which expresses a polypeptide capable of producing a measurable signal, coupled to, and under the control of, the promoter, under conditions such that the chemical if capable of acting as a transcriptional modulator of the gene-of-interest, causes a measurable signal to be produced by the polypeptide expressed by the reporter gene; (b) measuring the amount of the signal so produced; (c) comparing the amount of signal so measured with the amount of signal measured in the absence of any chemical being tested and upon contacting the sample with other chemicals so as to thereby identify the chemical as a chemical which causes a change in the amount of signal produced by the polypeptide, and thus as a chemical capable of transcriptionally modulating the expression of the gene-of-interest.

58. A method of determining whether a chemical not previously known to be a modulator of protein biosynthesis is capable of specifically transcriptionally modulating the expression of a gene-of-interest which includes:

(a) contacting a sample which contains a predetermined number of identical eucaryotic reporter cells with a predefined concentration of the chemical to be tested, each such reporter cell being a transfected parental cell including a single DNA construct consisting essentially of in 5' to 3' order (i) a modulatable transcriptional regulatory sequence of the gene-of-interest, (ii) a promoter of the gene-of-interest, and (iii) a DNA sequence transcribable into mRNA coupled to

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and under the control of, the promoter, under conditions such that the chemical if capable of acting as a transcriptional modulator of the gene-of-interest, causes a measurable difference in the amount of mRNA transcribed from the DNA sequence;

(b) measuring the amount of the mRNA so produced;

(c) comparing the amount so measured with the amount of mRNA measured in the absence of any chemical being tested and upon contacting the sample with other chemicals so as to thereby identify the chemical as a chemical which causes a change in the amount of mRNA, and thus identifying the chemical as a chemical capable of transcriptionally modulating the expression of the gene-of-interest.

59. A method of any one claims 57 or 58, wherein the molecule (a) does not naturally occur in the cell, (b) specifically transcriptionally modulates expression of the gene-of-interest, and (c) binds to DNA or RNA or binds to a protein through a domain of such protein which is not a ligand

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binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand binding domain is normally associated with a defined physiological or pathological effect.

60. A method of any of claims 57 or 58, wherein the sample ~~comprises~~ includes cells in monolayers.

61. A method of any of claims 57 or 58, wherein the sample ~~comprises~~ includes cells in suspension.

62. A method of any of claims 57 or 58, wherein the cells ~~comprise~~ include human, animal, or plant cells.

63. A method of any of claims 57 or 58, wherein the cells are bacterial cells.

64. A method of any of claims 57 or 58, wherein the cells are fungal cells.

65. A method of any of claims 57 or 58, wherein the predefined number of cells is from about 1 to about 5×10^5 cells.

66. A method of claim 65, wherein the predefined number of cells is from about 2×10^2 to about 5×10^4 cells.

67. A method of any of claims 57 or 58, wherein the predetermined amount of the molecule to be tested is based upon the volume of the sample.

68. A method of any of claims 57 or 58, wherein the predetermined amount is from about 1.0pM to about 20 μM.



69. A method of any of claims 57 or 58, wherein the predetermined amount is from about 10nM to about 500μM.

70. A method of any of claims 57 or 58, wherein the contacting is effected from about 1 hour to about 24 hours.

71. A method of claim 70, wherein the contacting is effected from about 2 to about 12 hours.

72. A method of any of claims 57 or 58, wherein the contacting is effected with more than one predetermined amount of the molecule to be tested.

73. A method of any of claims 57 or 58, wherein the molecule to be tested is a purified molecule.

74. A method of any of claims 57 or 58, wherein the modulatable transcriptional regulatory sequence comprises a cloned genomic regulatory sequence.

75. A method of any of claims 57 or 58, wherein the DNA consists essentially of more than one modulatable transcriptional regulatory sequence.

76. A method of claim 57, wherein the reporter gene is inserted downstream of the promoter of the gene-of-interest by homologous recombination.

77. A method of claim 57, wherein the reporter gene encodes a luciferase.

78. A method of claim 57, wherein the reporter gene encodes chloramphenicol acetyltransferase.

79. A method of claim 57, wherein the reporter gene encodes β glucuronidase.

80. A method of claim 57, wherein the reporter gene encodes β galactosidase.

81. A method of claim 57, wherein the reporter gene encodes neomycin phosphotransferase.

82. A method of claim 57, wherein the reporter gene encodes guanine xanthine phosphoribosyltransferase.

83. A method of claim 58, wherein mRNA is detected by quantitative polymerase chain reaction.

84. A screening method according to any of claims 57 or 58 which comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells, under conditions such that contacting is effected with a predetermined amount of each different molecule to be tested.

85. A screening method of claim 84, wherein the plurality of samples comprises more that about 10^6 samples.

86. A screening method of claim 84, wherein the plurality of samples comprises more than about 6 X 10^6 samples.

87. A method of essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating one or more genes of interest in a panel of such genes which

~~comprises~~ includes essentially simultaneously screening the molecules against each of the genes of interest according to the method of claim 84.

88. A screening method of any of claims 86 or 87, wherein more that about 10^3 samples per week are contacted with different molecules.

89. A method for transcriptionally modulating in a multicellular organism the expression of a gene-of-interest, the expression of which is associated with a defined physiological or pathological effect in the organism, which ~~comprises~~ includes administering to the organism an amount of a molecule effective to transcriptionally modulate expression of the gene and thus affect the defined physiological or pathological effect, which molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the gene-of-interest, and (c) binds to DNA or RNA or binds to a protein through a domain of such protein which is not a ligand binding domain of a receptor which naturally occurs in the organism, the binding of a ligand to which ligand binding domain is normally associated with the defined physiological or pathological effect.

90. A method of claim 89, wherein the multicellular organism is a human being.

91. A method of claim 89, wherein the multicellular organism is an animal.

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92. A method of claim 89, wherein the multicellular organism is a plant.

93. A method of claim 89, wherein the defined pathological effect is a disorder and modulated expression of the gene-of-interest is associated with amelioration of the disorder.

94. A method of claim 90, wherein the defined pathological effect is a disorder selected from the group consisting of cancer, a hematopoietic dysfunction diabetes, tissue inflammation, atherosclerosis, viral infections, dysfunctions of memory or learning, and dysfunctions in a cholesterol or other metabolic pathway.

95. A method of claim 91, wherein the defined physiological effect is growth and the organism is an animal such as a man, a cow, a pig, a bird, a fish, a sheep or a horse.

96. A method of claim 92, wherein the defined physiological or pathological effect is an agronomically important trait.

97. A method of claim 90 or 91, wherein the administering ~~comprises~~ includes topical contact.

98. A method of claim 90 or 91, wherein the administering ~~comprises~~ includes oral, transdermal, intravenous, intramuscular or subcutaneous administration.

99. A method of any of claims 57, 58, or 89, wherein the gene-of-interest encodes a naturally occurring

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receptor.

100. A method of claim 99, wherein the receptor is a testosterone receptor.

101. A method of claim 99, wherein the receptor is an estrogen receptor.

102. A method of any of claims 57, 58, or 89, wherein the receptor which naturally occurs in the cell is a testosterone receptor.

103. A method of any of claims 57, 58, or 89, wherein the receptor which naturally occurs in the cell is an estrogen receptor.

104. A method of any of claims 57, 58, or 89, wherein the gene-of-interest encodes a TGF-β receptor.

105. A method of any of claims 57, 58, or 89, wherein the gene-of-interest encodes TGF-β1.

106. A method of any of claims 57, 58, or 89, wherein the gene-of-interest encodes TGF-β2.

107. A method of any of claims 57, 58, or 89, wherein the gene-of-interest encodes TGF-β-3.

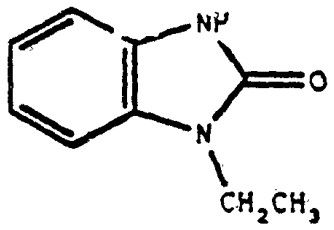
108. A method of any of claims 57, 58, or 89, wherein the gene-of-interest encodes an oncogene.

109. A method of claim 108, wherein the oncogene is the neu oncogene.

110. A method of claim 108, wherein the oncogene is

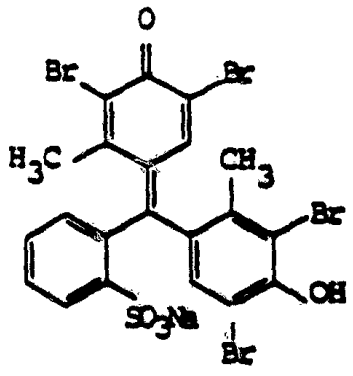
selected from the group consisting of H-, N-, and K-ras oncogenes.

111. A method for enhancing the expression of human growth hormone by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:



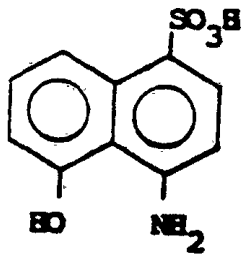
effective to enhance the expression of human growth hormone by the cell.

112. A method for enhancing the expression of human growth hormone by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing contacting the cell with an amount of a molecule having the structure:



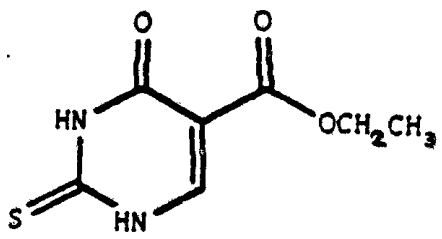
effective to enhance the expression of human growth hormone by the cell.

113. A method for enhancing the expression of human growth hormone by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:



effective to enhance the expression of human growth hormone by the cell.

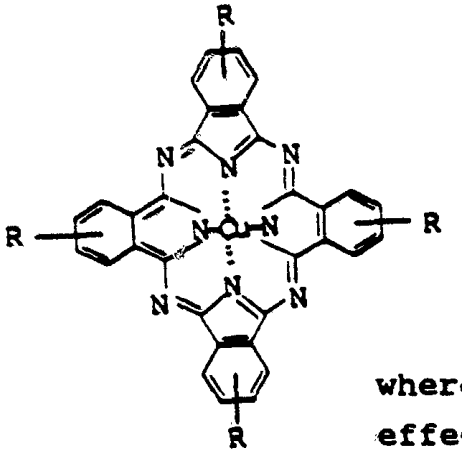
114. A method for enhancing the expression of human growth hormone by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, human growth hormone, comprising contacting the cell with an amount of a molecule having the structure:

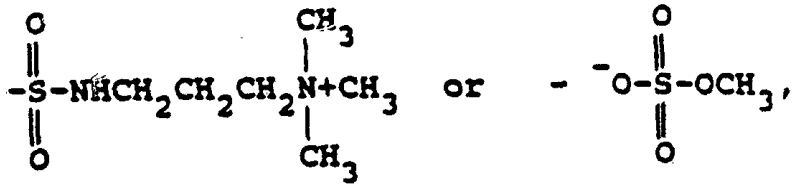


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effective to enhance the expression of human growth hormone by the cell.

115. A method for enhancing the expression of human growth hormone by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, human growth hormone, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:

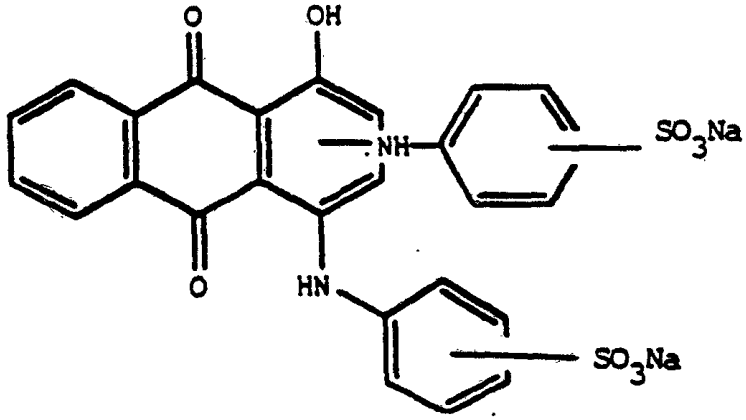




wherein R is

effective to enhance the expression of human growth hormone by the cell.

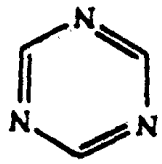
116. A method for enhancing the expression of G-CSF by a cell which (i) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:



effective to enhance the expression of G-CSF by the cell.

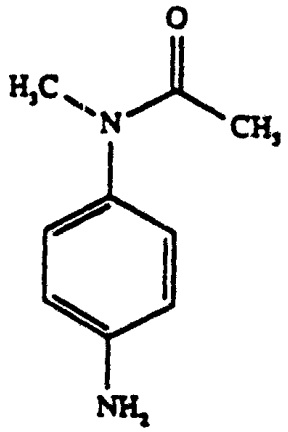
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117. A method for enhancing the expression of G-CSF by a cell which (i) comprises (includes) DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ (including) contacting the cell with an amount of molecule having the structure:



effective to enhance the expression of G-CSF by the cell.

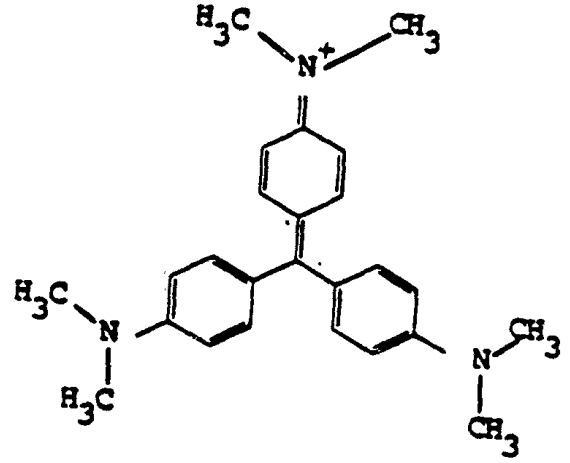
118. A method for enhancing the expression of G-CSF by a cell which (i) comprises (includes) DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ (including) contacting the cell with an amount of a molecule having the structure:



effective to enhance the expression of G-CSF by the cell.

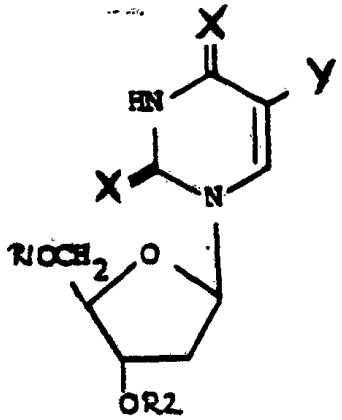
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119. A method for enhancing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:



effective to enhance the expression of G-CSF by the cell.

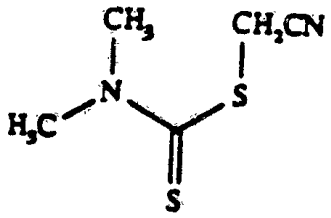
120. A method for enhancing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:



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wherein R1 is hydrogen or a lower alkyl group,
wherein R2 is hydrogen or a lower alkyl group,
wherein X is oxygen or $-NH_2$,
wherein Y is bromine, fluorine, chlorine, or iodine, and
which molecule is effective to enhance the expression of G-CSF by the cell.

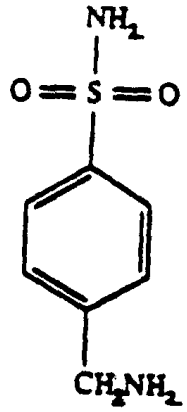
121. A method for enhancing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:



effective to enhance the expression of G-CSF by the cell.

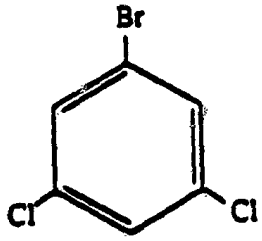
122. A method for enhancing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:

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effective to enhance the expression of G-CSF by the cell.

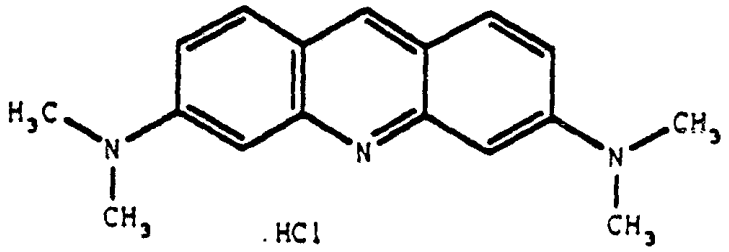
123. A method for enhancing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:



effective to enhance the expression of G-CSF by the cell.

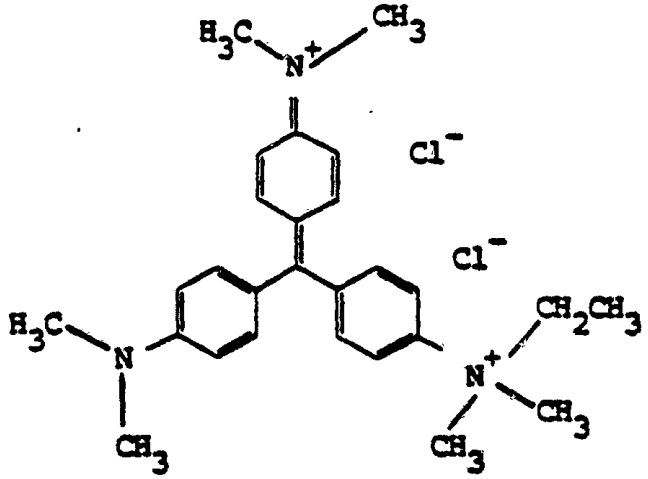
124. A method for enhancing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:

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effective to enhance the expression of G-CSF by the cell.

125. A method of decreasing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:

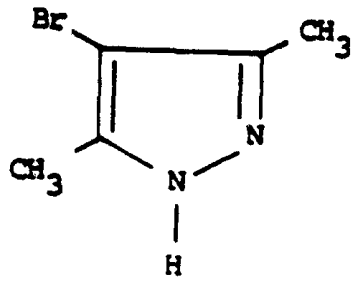


effective to decrease the expression of G-CSF by the human being.

126. A method for decreasing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii)

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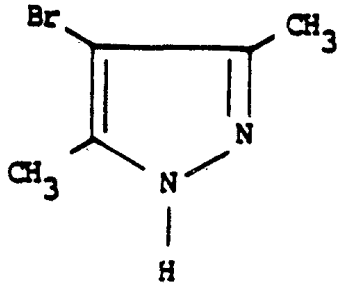
is capable of expressing, G-CSF, including ~~comprising~~ contacting the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the human being.

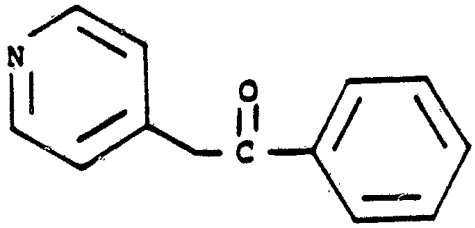
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127. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and effect the metabolic functions of neutrophils in, the human being.

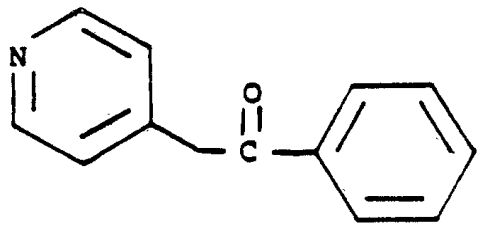
128. A method of decreasing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF

by the human being.

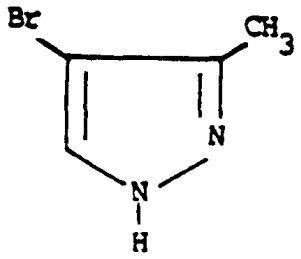
129. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and effect the metabolic functions of neutrophils in, the human being.

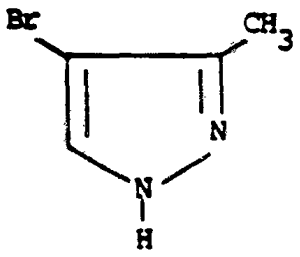
130. A method of decreasing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:

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effective to decrease the expression of G-CSF by the human being.

131. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including administering to the human being an amount of a molecule having the structure:

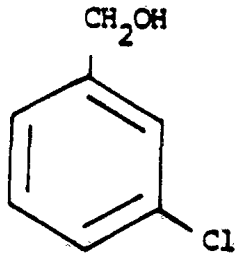


effective to decrease expression of G-CSF by, and thus decrease the formation and effect the metabolic functions of neutrophils in, the human being.

132. A method of decreasing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting

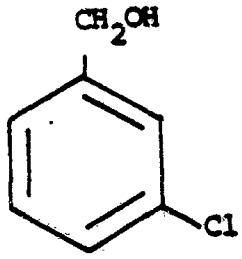
AUSTRALIAN PATENT OFFICE

the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the human being.

133. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including administering to the human being an amount of a molecule having the structure:

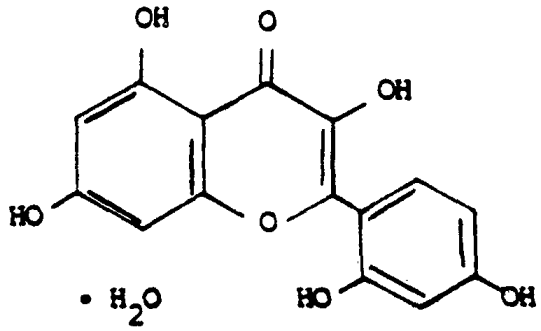


effective to decrease expression of G-CSF by, and thus decrease the formation and effect the metabolic functions of neutrophils in, the human being.

134. A method of decreasing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting

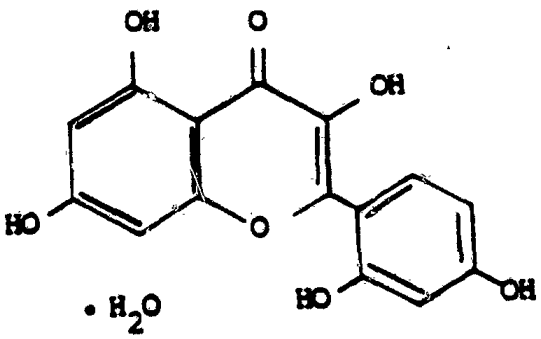
AUSTRALIAN PATENT OFFICE

the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the human being.

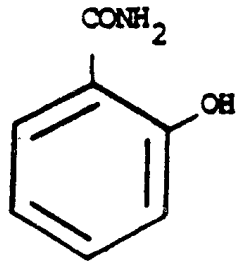
135. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and effect the metabolic functions of neutrophils in, the human being.

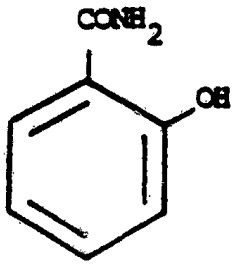
AUSTRALIAN PATENT OFFICE OK

136. A method of decreasing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the human being.

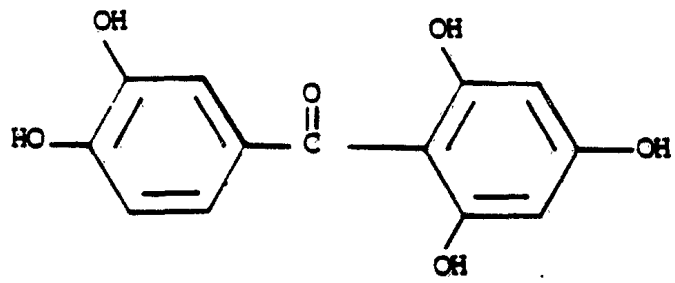
137. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including administering to the human being an amount of a molecule having the structure:



AUSTRALIAN PATENT OFFICE

effective to decrease expression of G-CSF by, and thus decrease the formation and effect the metabolic functions of neutrophils in, the human being.

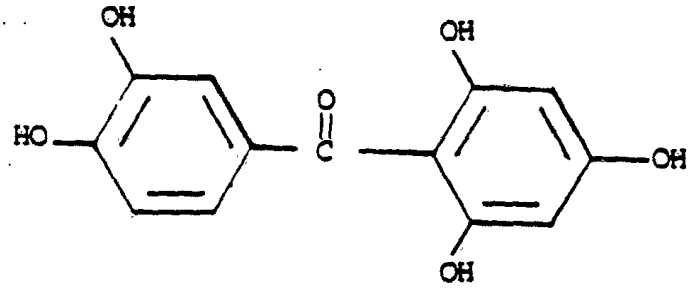
138. A method of decreasing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:



effective to decrease the expression of G-CSF by the human being.

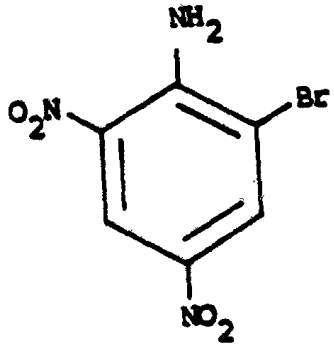
AUSTRALIAN PATENT OFFICE

139. A method of decreasing the formation of neutrophils and affecting the metabolic functions of neutrophils in a human being who (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including administering to the human being an amount of a molecule having the structure:



effective to decrease expression of G-CSF by, and thus decrease the formation and effect the metabolic functions of neutrophils in, the human being.

140. A method of decreasing the expression of G-CSF by a cell which (i) ~~comprises~~ includes DNA encoding, and (ii) is capable of expressing, G-CSF, ~~comprising~~ including contacting the cell with an amount of a molecule having the structure:



AUSTRALIAN PATENT OFFICE

effective to decrease the expression of G-CSF by the human being.

141. A method of transcriptionally modulating the expression of a gene-of-interest substantially as hereinbefore described with reference to the Examples.

DATED: 21 April, 1995

PHILLIPS, ORMONDE & FITZPATRICK

Attorneys for:

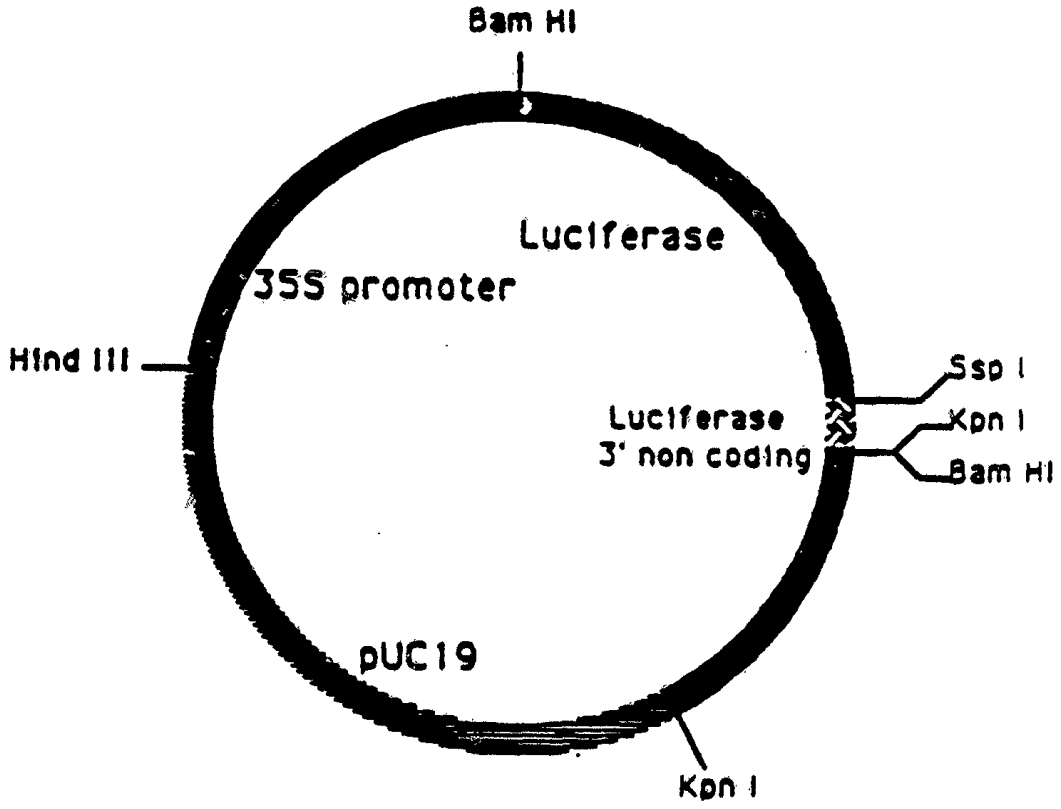
ONCOGENE SCIENCE, INC.

David B Fitzpatrick

AUSTRALIAN PATENT OFFICE

SC C:\WINWORD\SIMONE\EPNODEL\61400D10.DOC

Figure 1. pDO432



pDO432

Figure 2. pSVLuc1

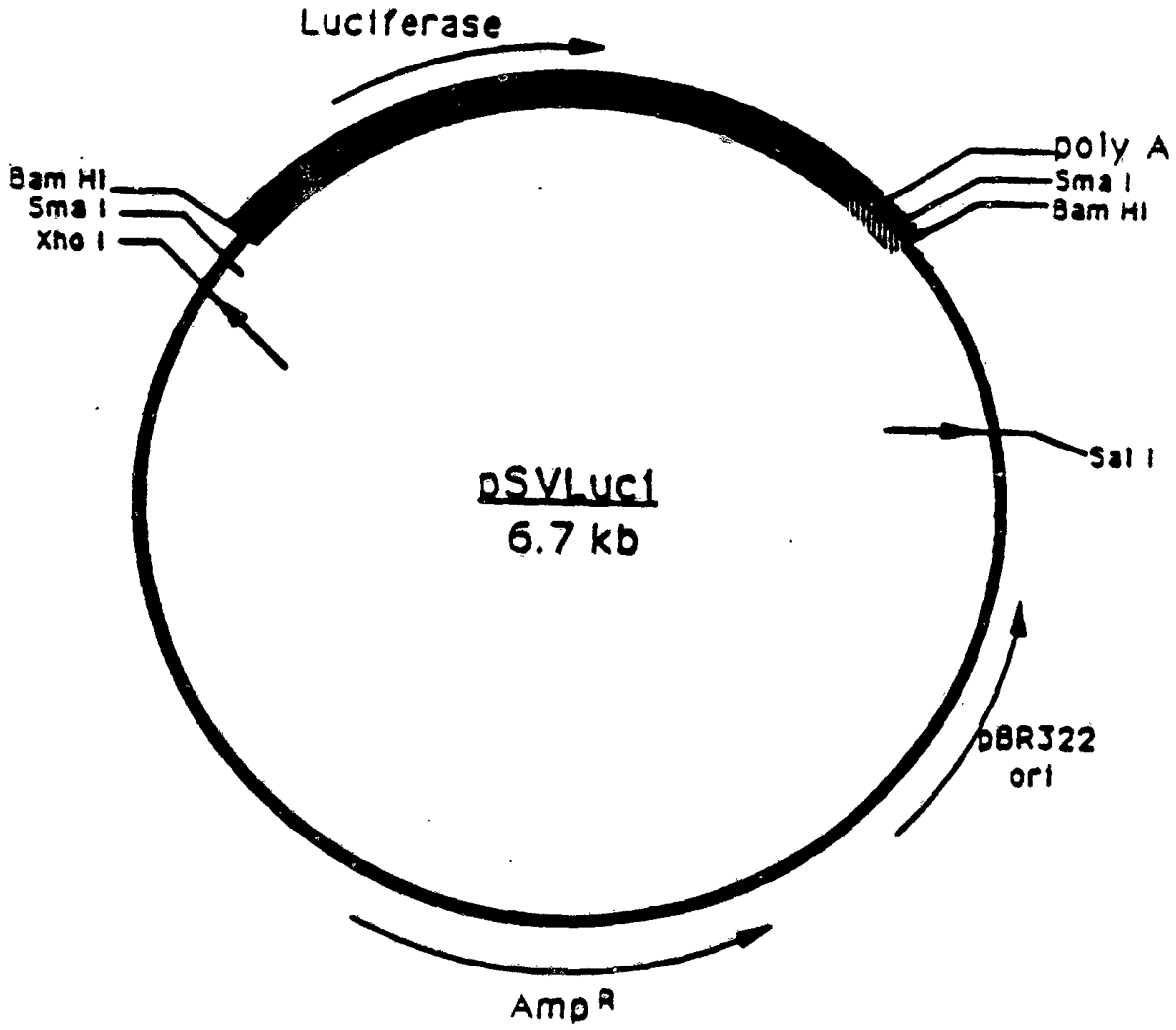


Figure 3. pMLuc1

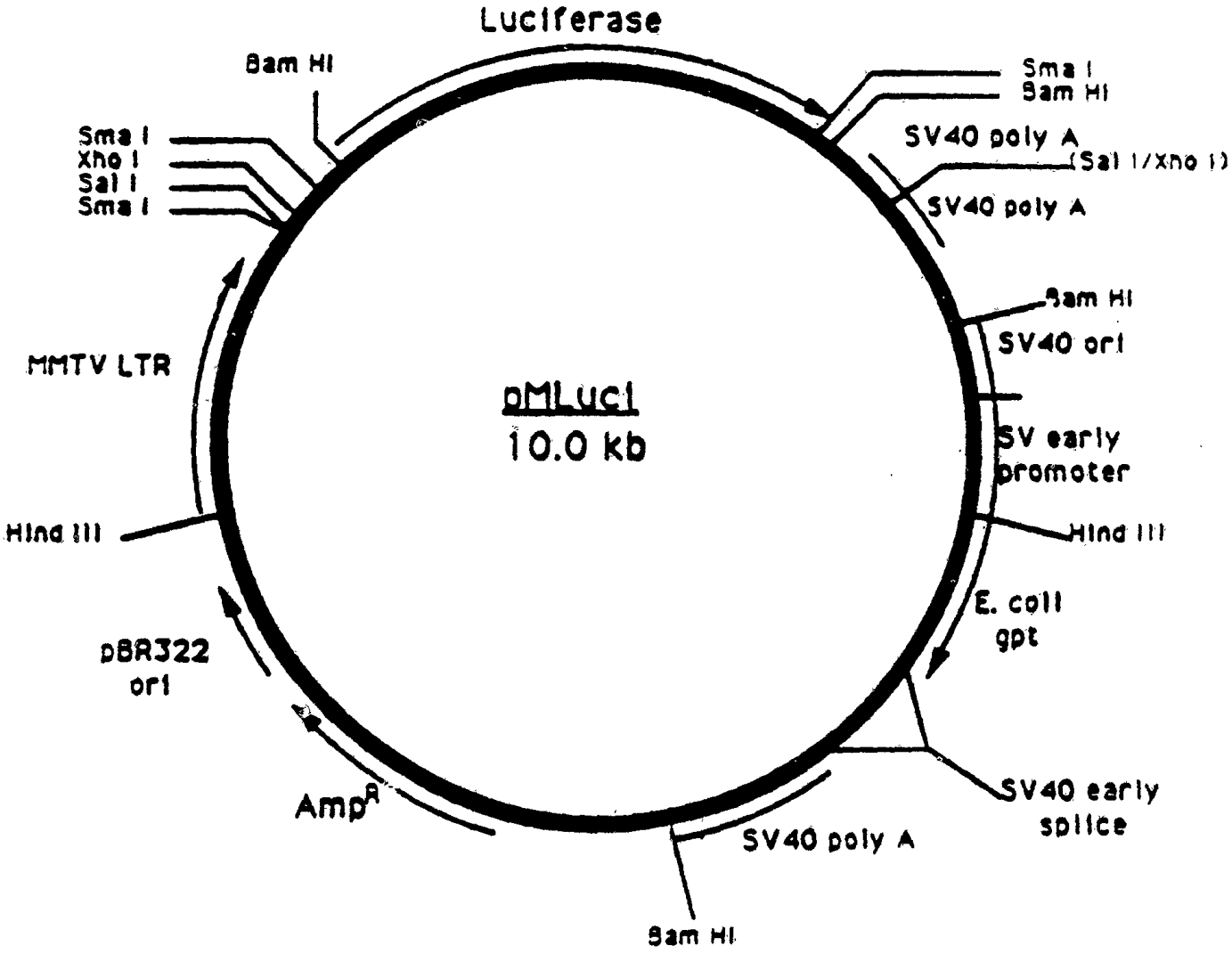


Figure 4. pUXLuc1

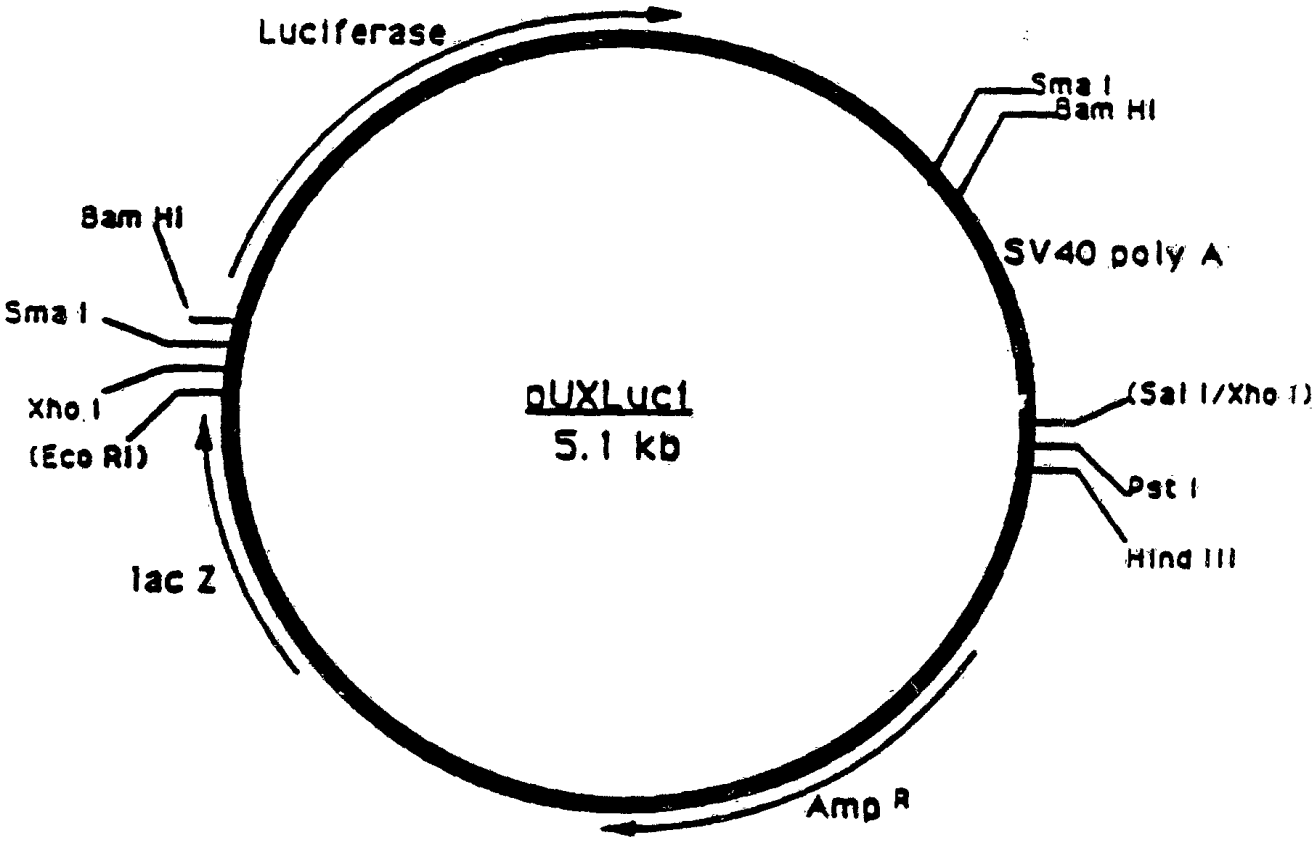


Figure 5. phGH-CAT

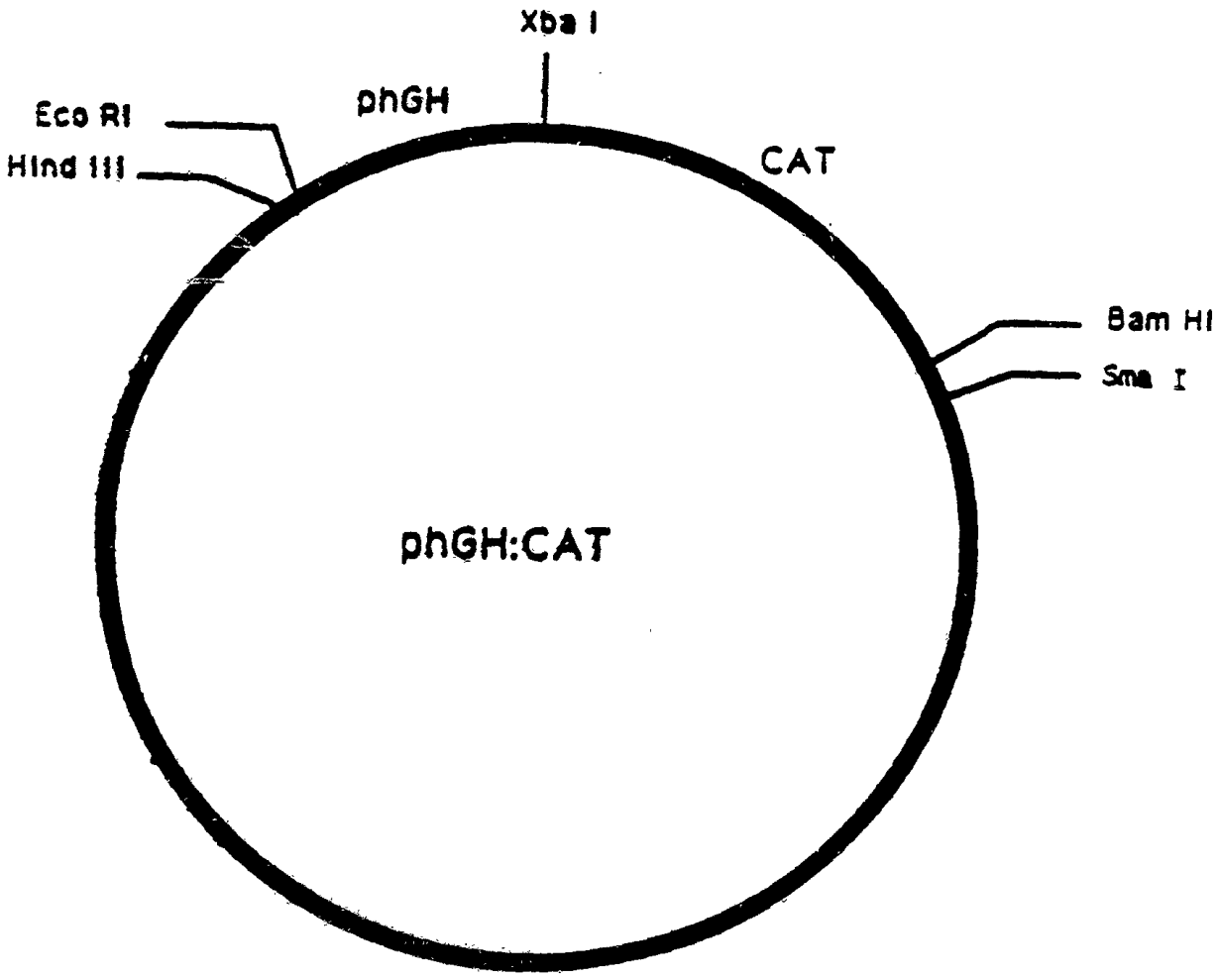


Fig. phGH-Luc1

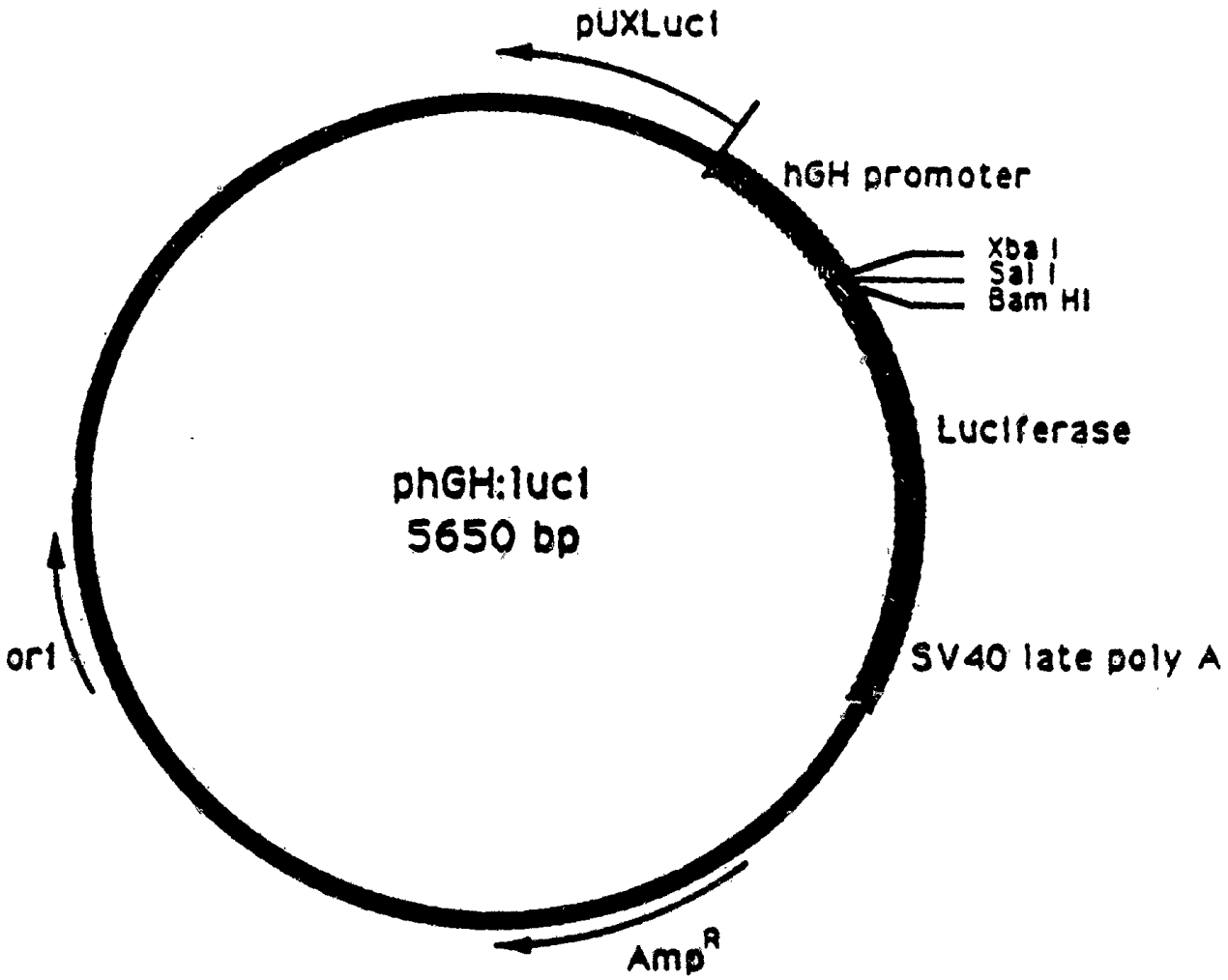


Figure 7. pJM710

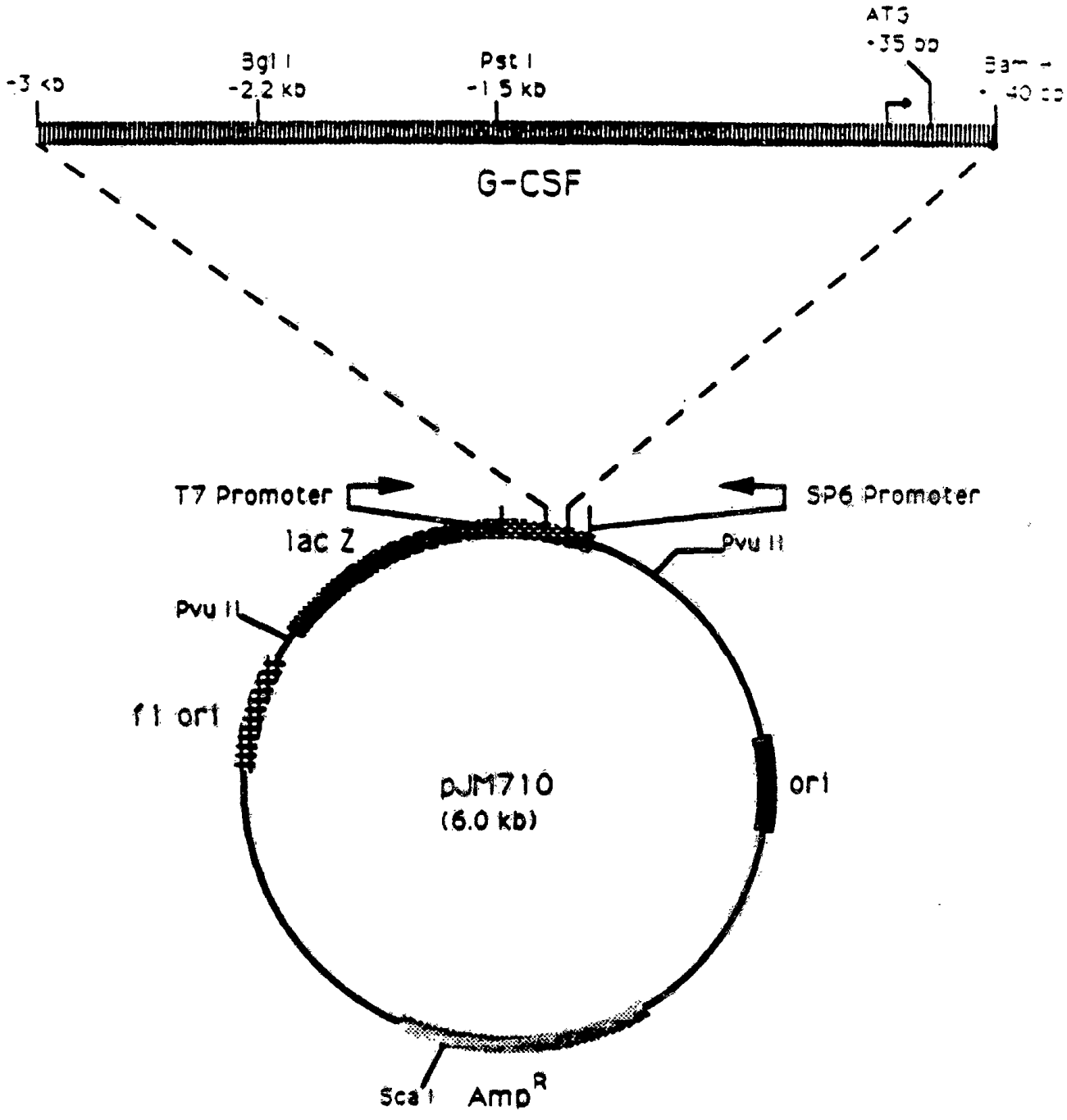


Figure 8. pGEM5-Luc1

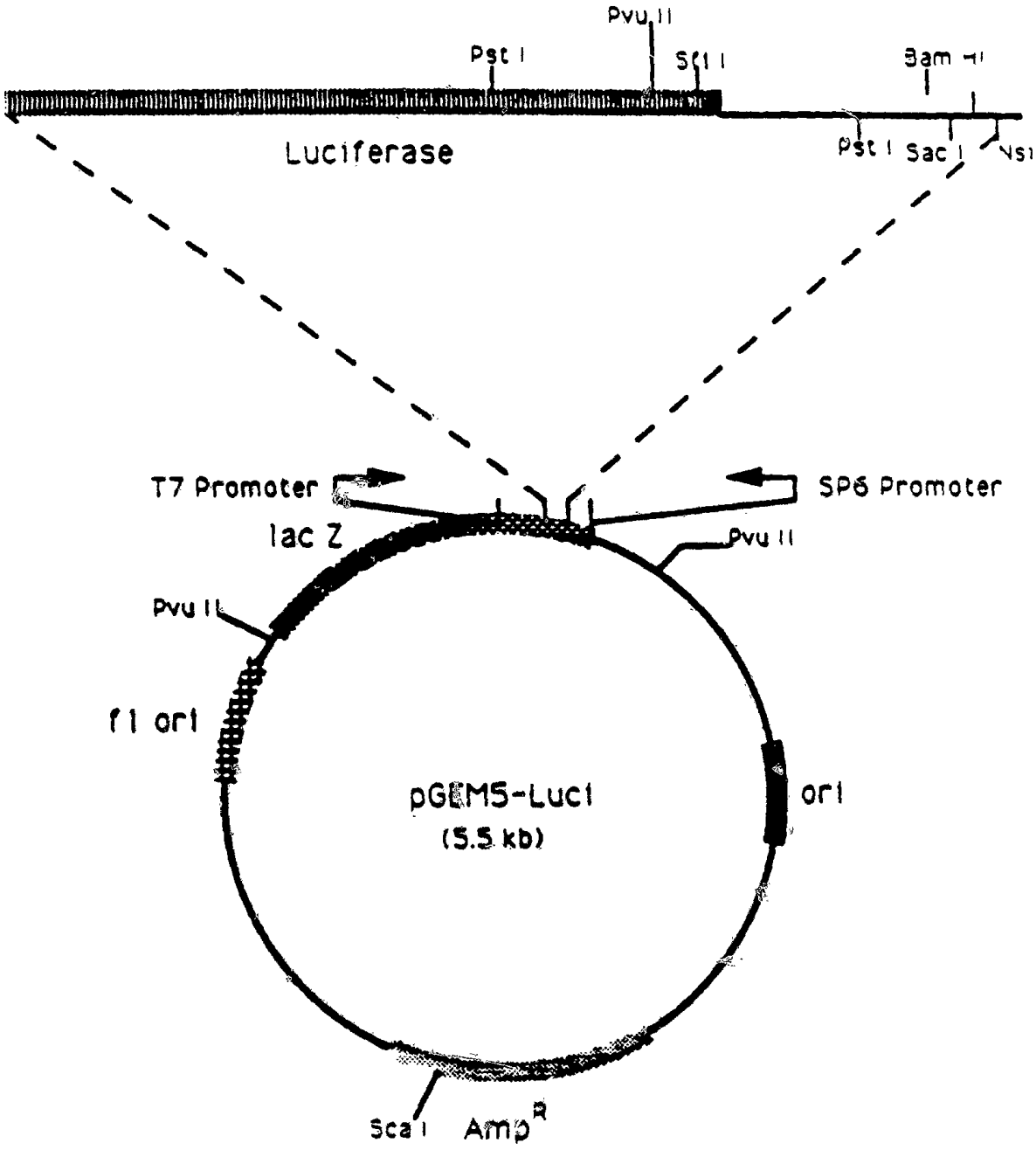


Figure 9. pG-Luc 1

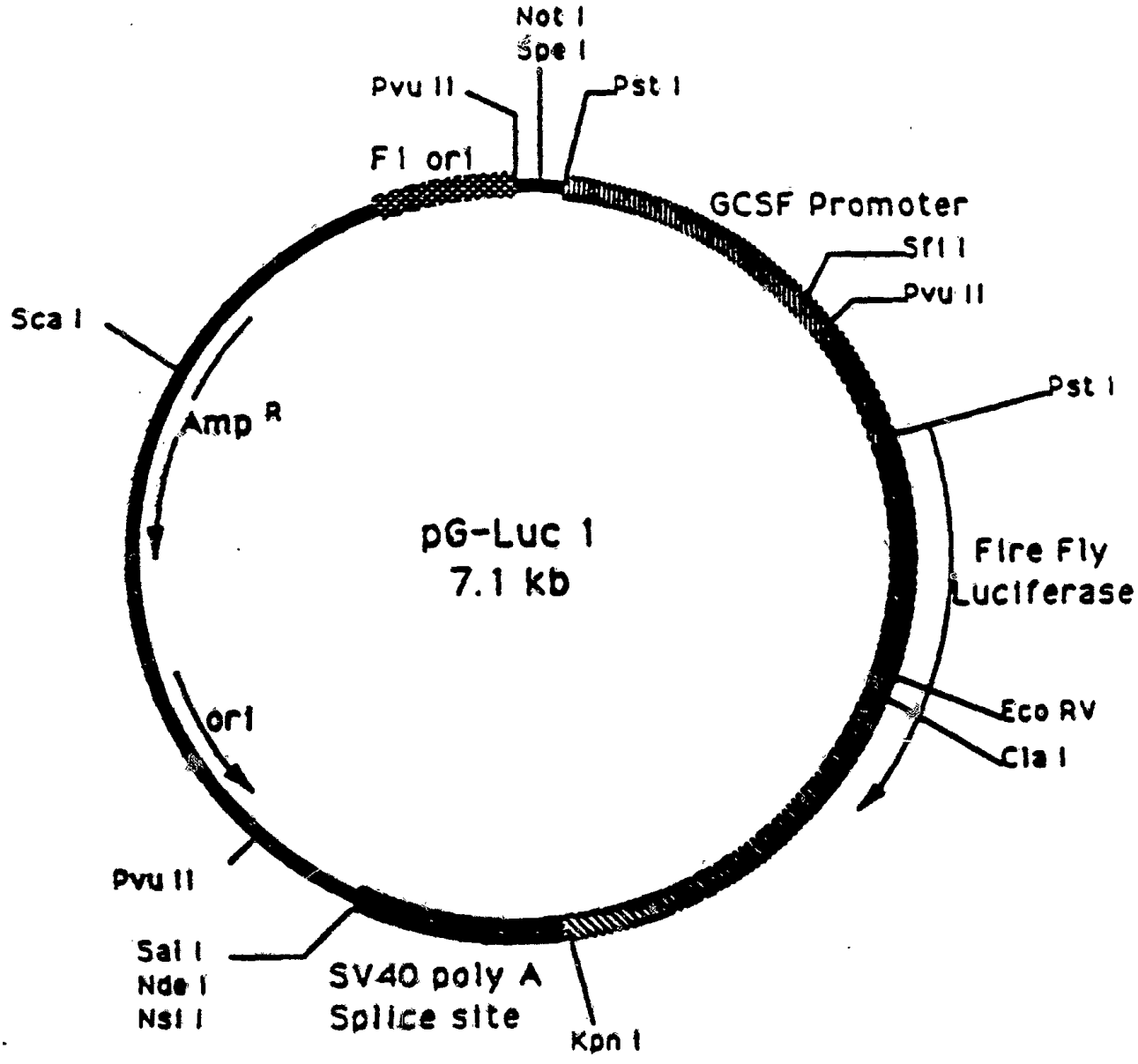


Figure 10. Response of Reporter Cell Clones To Known Inducers

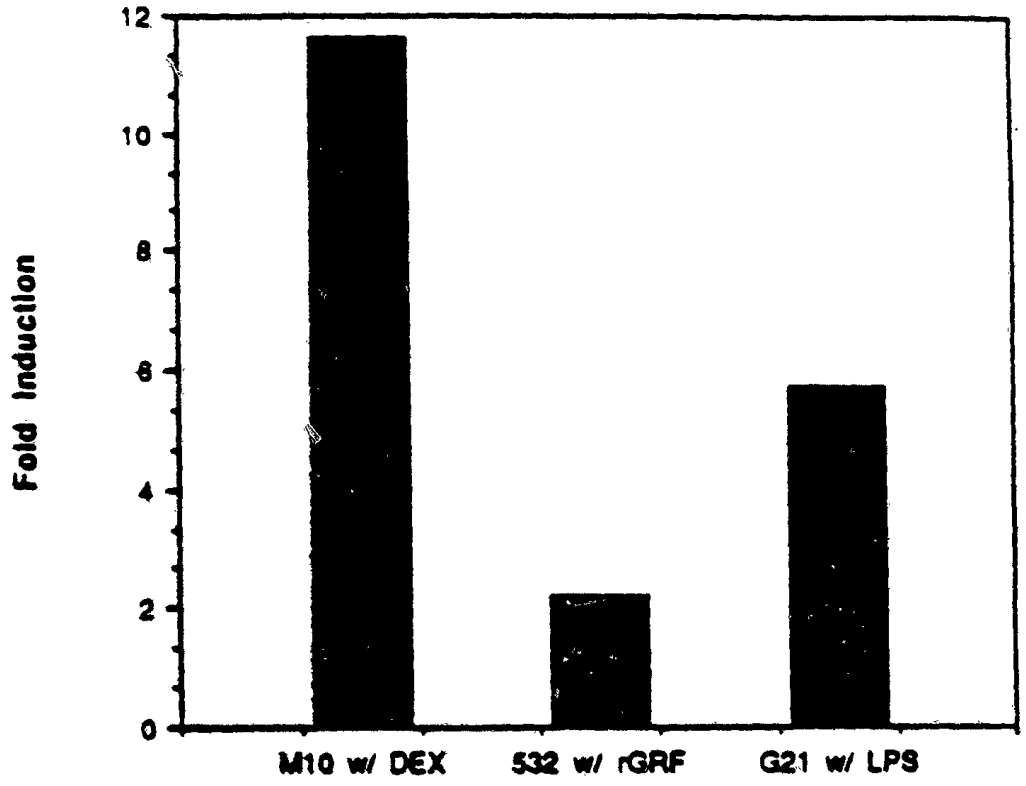


Figure 11 . Effects of Steroids on Clone M10

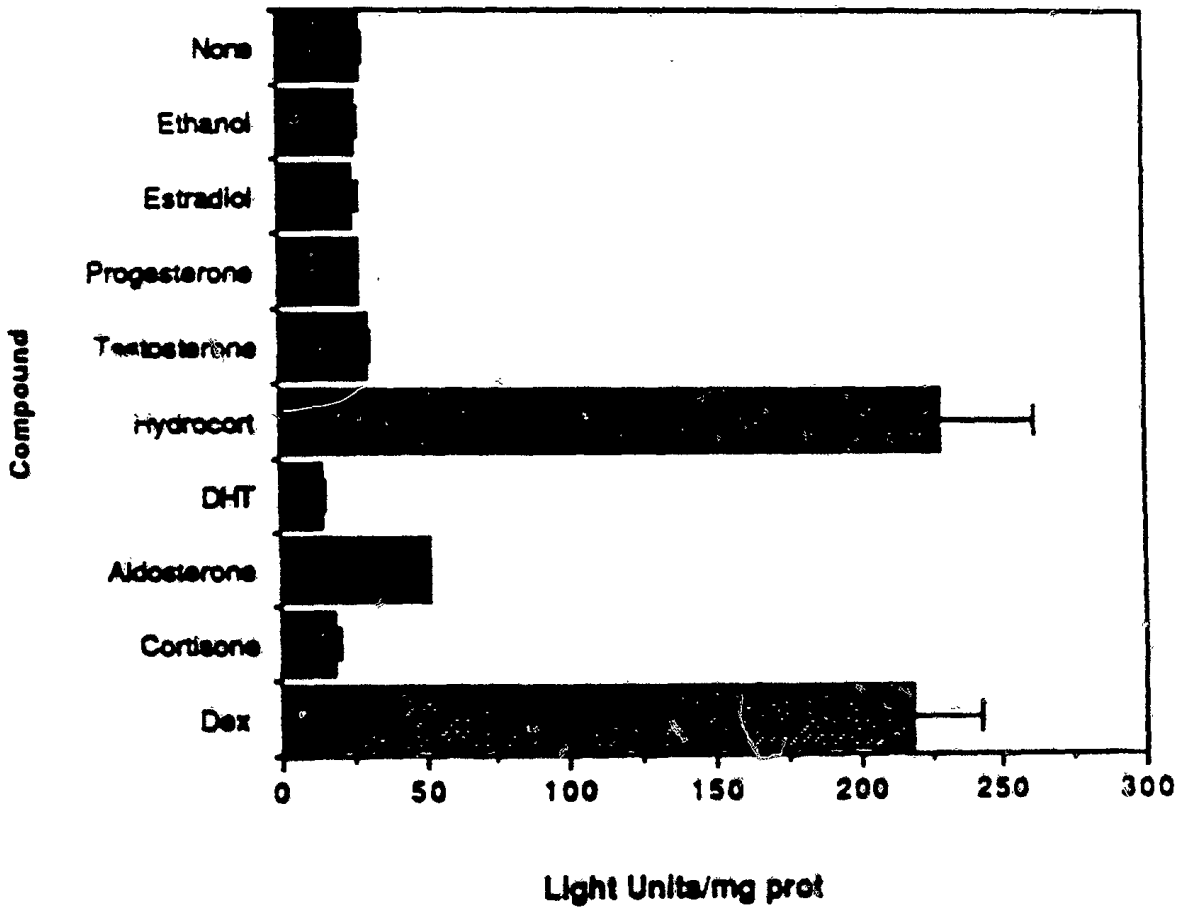


Figure 12

EXAMPLES OF PRIMARY SCREEN LEAD CHEMICALS—
SPECIFIC TRANSCRIPTIONAL INDUCERS

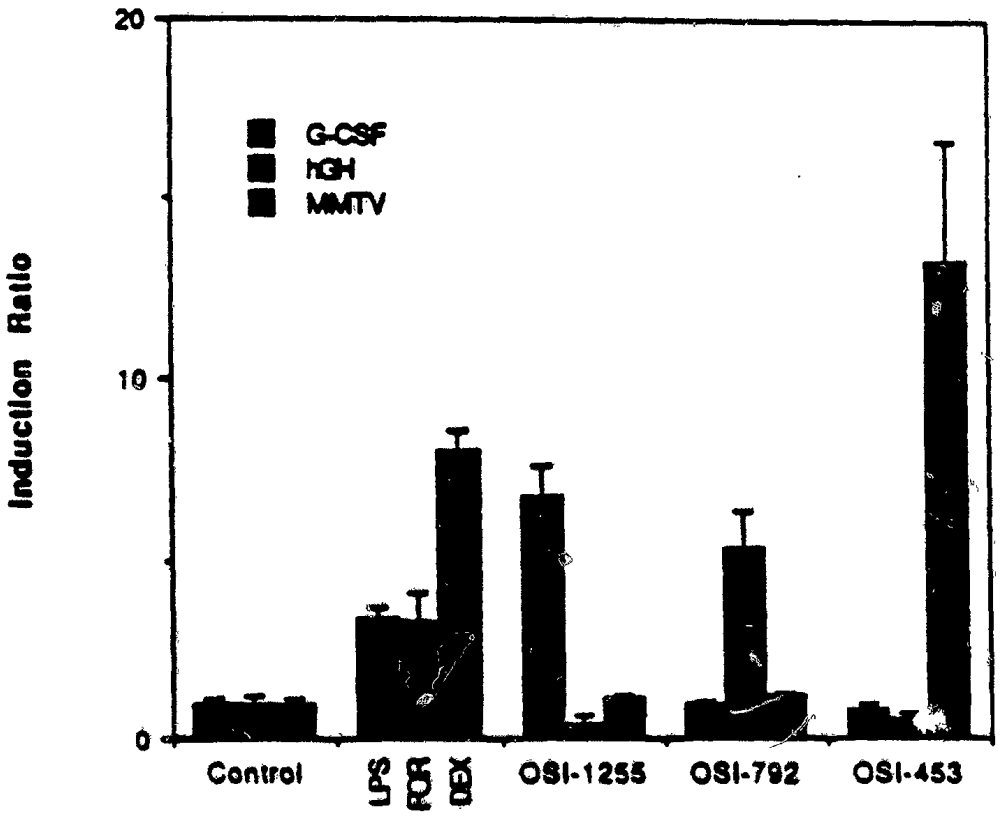


Figure 13

EXAMPLES OF PRIMARY SCREEN LEAD CHEMICALS
SPECIFIC TRANSCRIPTIONAL INHIBITORS

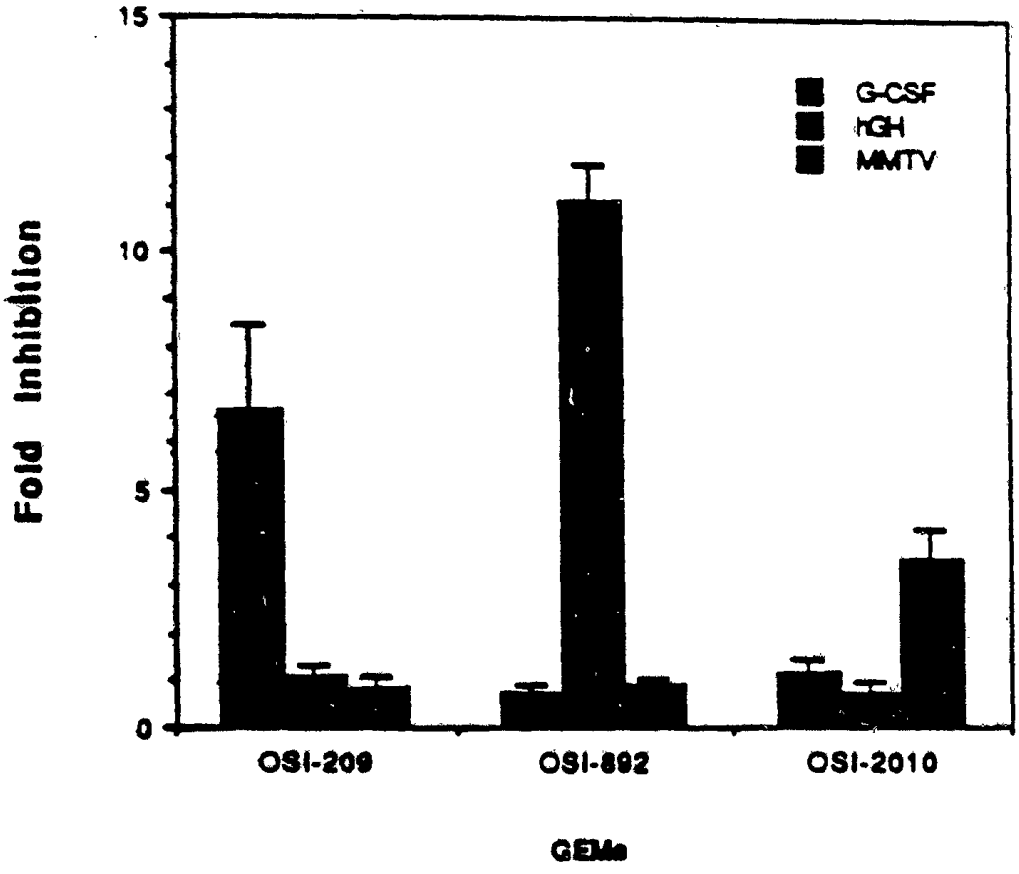


Figure 14

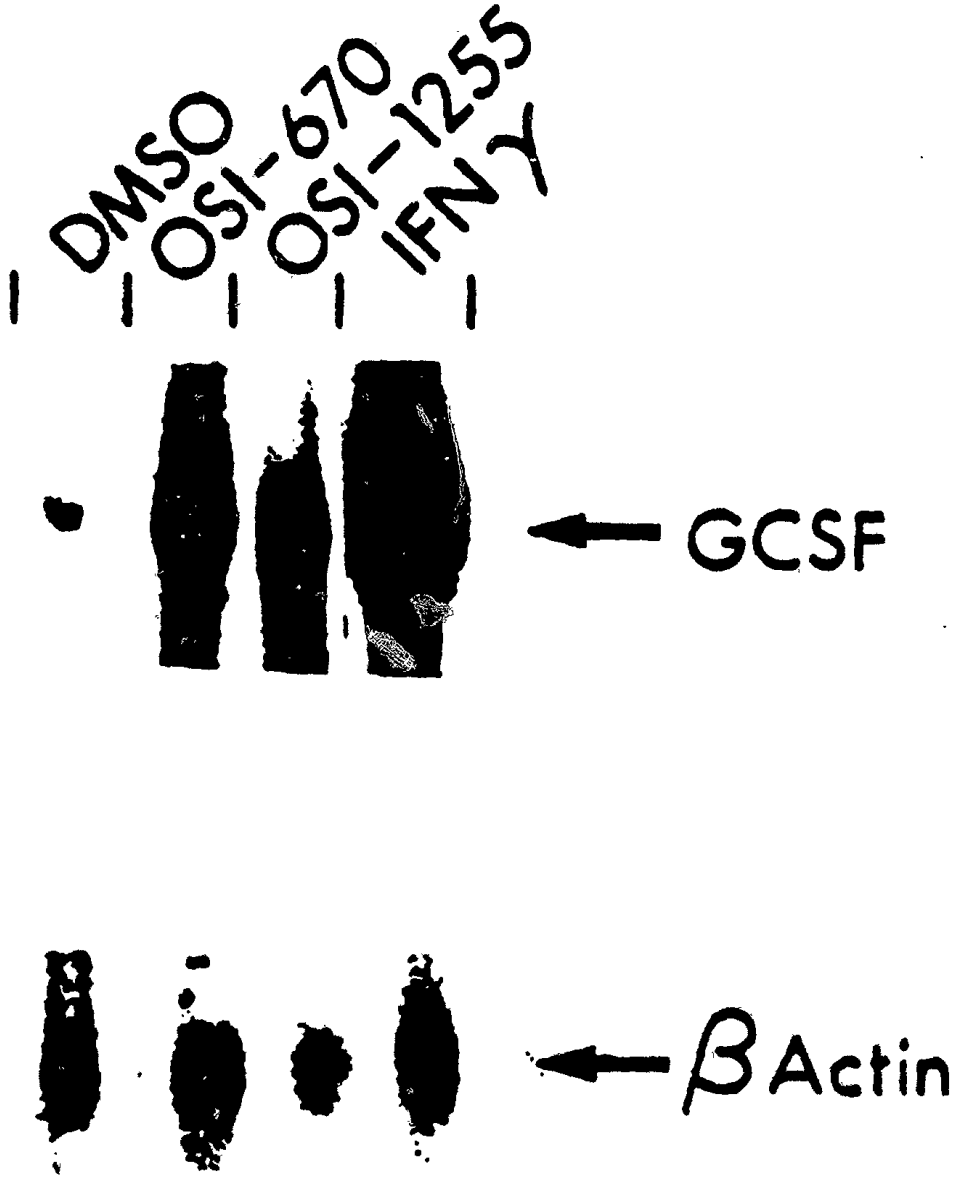


Figure 15

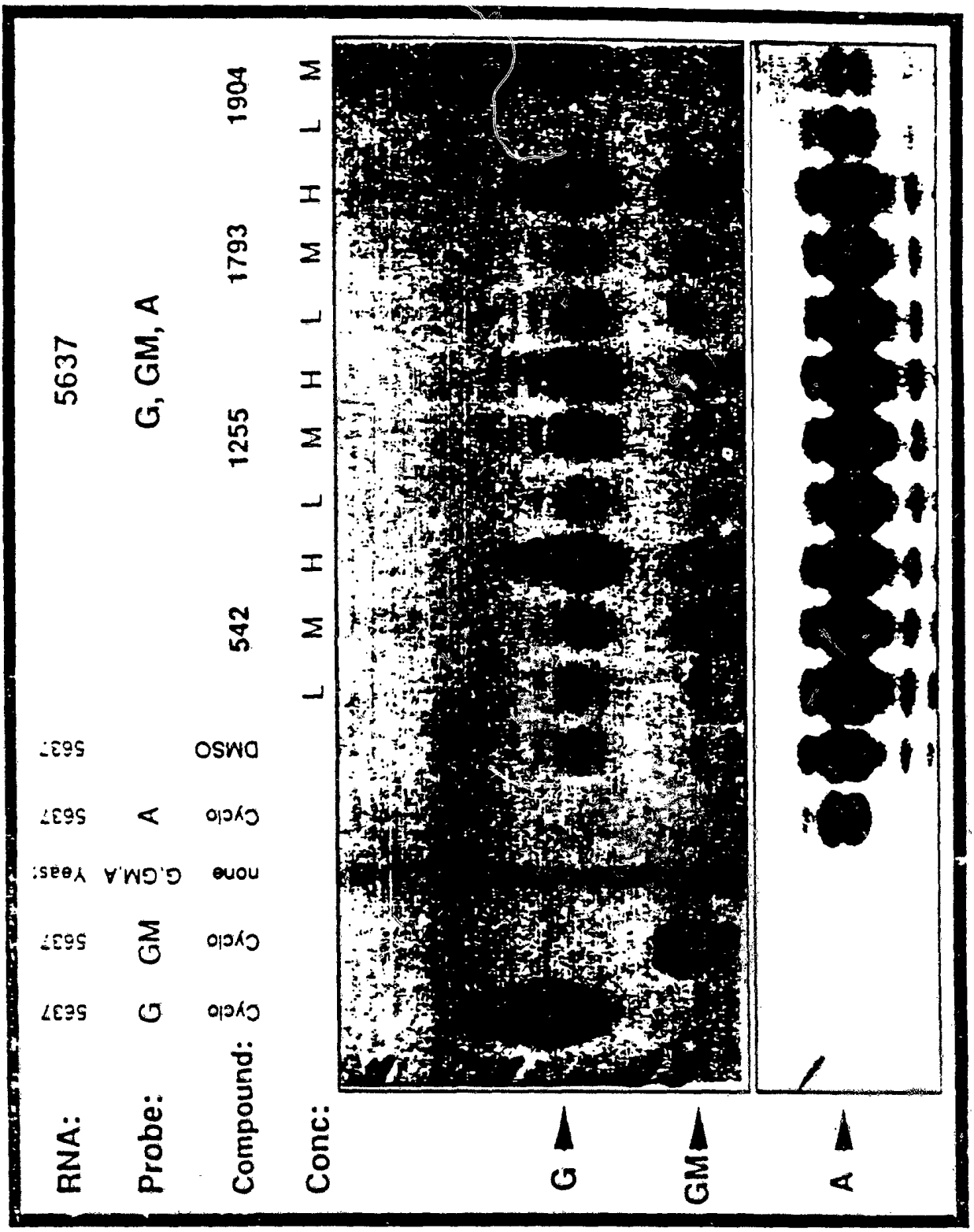
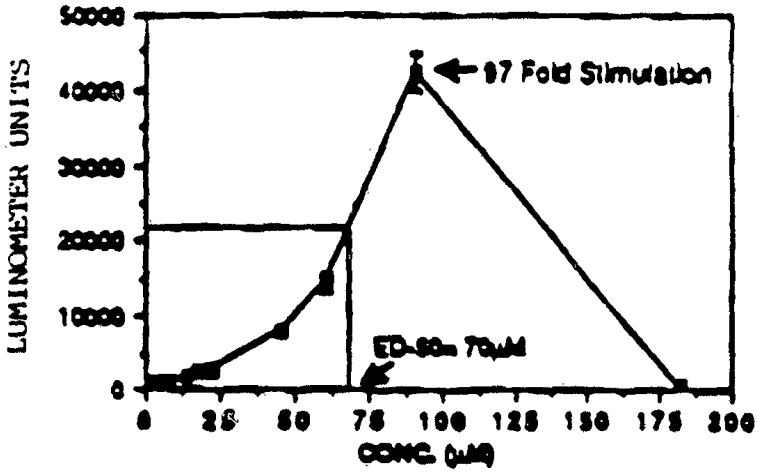
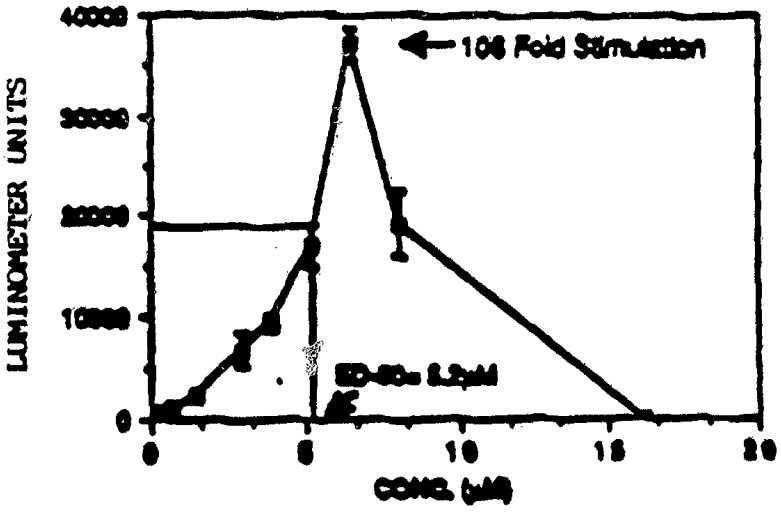

RNA: 5637
Probe: G GM A G, GM, A
Compound: Cyclo Cyclo none DMSO
Yeast: 5637 5637 G,GM,A 5637
Conc: L M H L M H L M H L M
542 1255 1793 1904
G
GM
A

Figure 16

DOSE RESPONSE ANALYSIS OF CMPD 670



DOSE RESPONSE ANALYSIS OF CMPD 1780



DOSE RESPONSE ANALYSIS OF CMPD 80

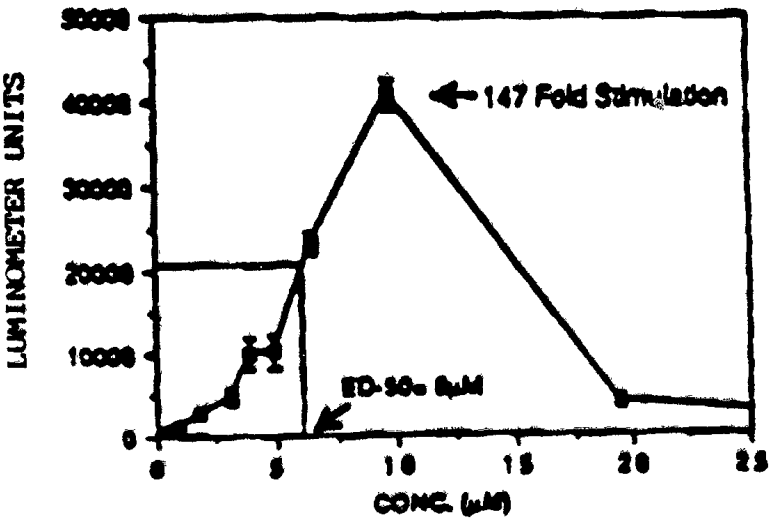


Figure 17

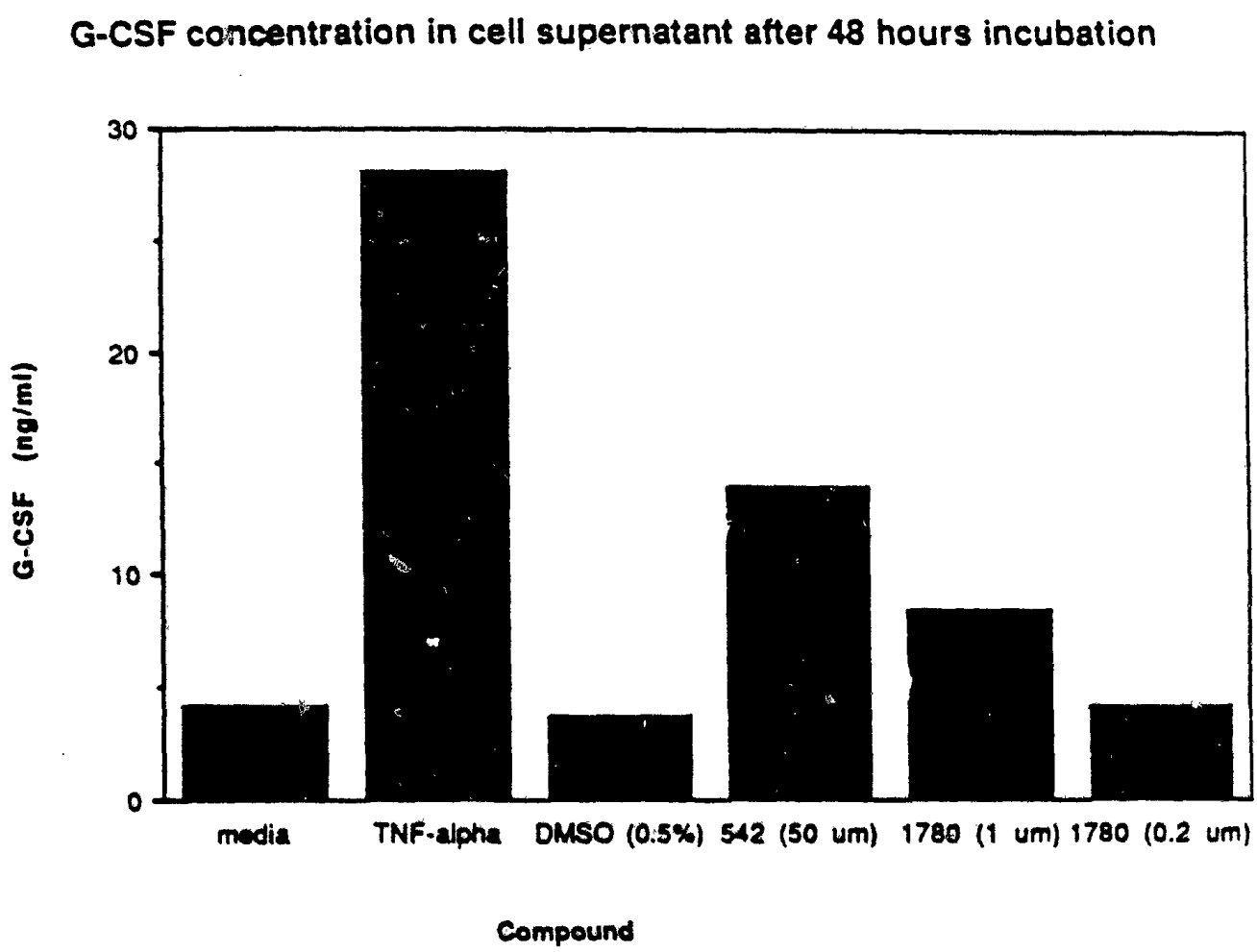
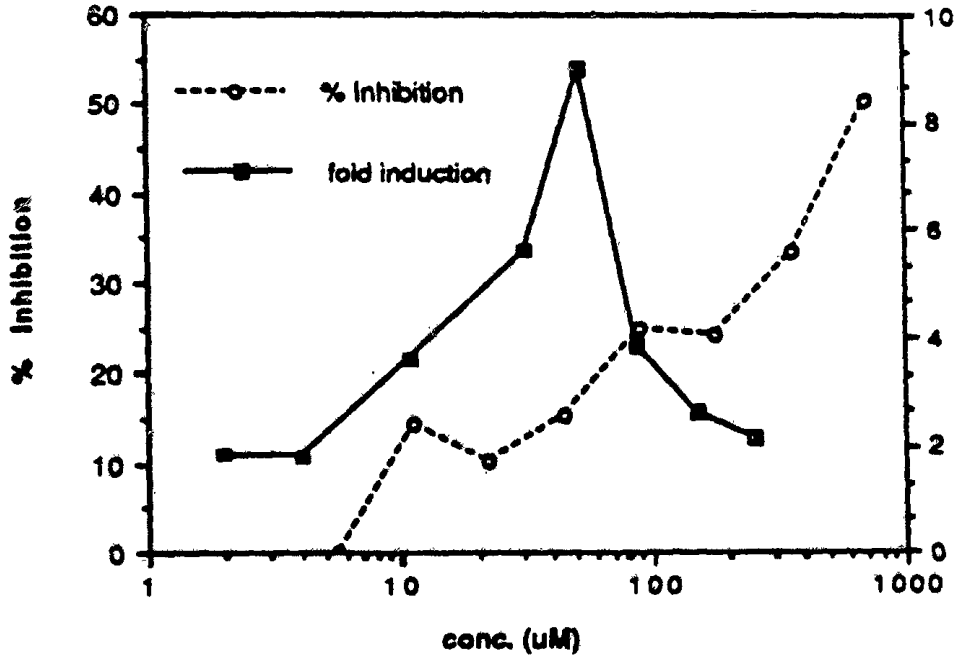

G-CSF concentration in cell supernatant after 48 hours incubation
G-CSF (ng/ml)
30
20
10
0
media
TNF-alpha
DMSO (0.5%)
542 (50 um)
1780 (1 um)
1780 (0.2 um)
Compound

Figure 18

542 - promoter stimulation versus respiratory inhibition



INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04021

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P 21/00,21/02; C12N 7/04,5/00,15/00,1/38; C12Q 1/66

USA: 435/69.5,69.62,69.4,70.1,236,240.2,172.1,172.2,172.3,244

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	435/8,69.4,69.5,69.62,70.1,172.1,172.2,172.3,236,240.2,244

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

U.S. Patent and Trademark Office Automated patent System
DIALOG- BIOSIS PREVIEWS, CHINESE PATENT ABSTRACTS, CLAIMS™/U.S. PATENTS, INPADOC/FAMILY AND LEGAL STATUS, WORLD PATENTS INDEX (SEE ATTACHMENT)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	US, A, 4,738,922 (Haseltine et al.) 19 April 1988. See col. 1-10 and the examples.	1-6 1-7,12
X Y	US, A, 4,740,461 (Kaufman) 26 April 1988. See col. 1-16 and the examples.	1-6 1-7,12
X Y	EP, A, 0,117,058 (Levinson et al.) 29 August 1984. See at least the abstract and pages 5-18.	1-6 1-7,12
X Y	Mol. Cell. Biol. Volume 7, No.6, issued June 1987 (Angel et al.) "12-0-tetradecanoyl phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region pages 2256-2266. See the abstract and figures.	1-7 1-7,12

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
17 October 1990	02 JAN 1991
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Christopher Low

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Proc. Natl. Acad. Sci. USA, Volume 83, issued May 1986 (Kaushansky et al.) "Genomic cloning, characterization, multilineage growth-promoting activity of human granulocyte-macrophage colony-stimulating factor". Pages 3101-3105. See Figure 2, and page 3105. (See Attachment)	1-7,12-15

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ... , because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

(See Attachment)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-7 and 12-19

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to: Form PCT/ISA/210 second sheet
III. DOCUMENTS CONSIDERED TO BE RELEVANT

Y	EMBO J. Volume 6, No. 9, issued September 1987 (Lødner et al.) "Human CSF-1: gene structure and alternative splicing of mRNA". pages 2693-2698 See at least page 2693, 2696-2997.	1-7,12-14, 17
Y	EMBO J. Volume 6, No. 4, issued April 1987 (Lefevre et al.) "Tissue-specific expression of the humar growth hormone gene is conferred in part by the binding of a specific trans-acting factor". pages 971-981. See at least page 971.	1-7, 12
Y	Proc. Natl. Acad. Sci. USA. Volume 82, issued November 1985 (Lin et al.) "Cloning and expression of the human erythropoietin gene". pages 7580-7584. See entire document.	1-7,12,13 18
Y	EMBO J. Volume 5, No. 3, issued March 1986, (Nagata et al.) "The chromosoaml gene structure and two mRNAs for human granulocyte colony-stimulating factor. pages 575-581. See at least the abstract.	1-7,12-14 16
Y	Cell Volume 47, issued 10 October 1986, (Yang et al.) "Human IL-3 (multi-CSF): Identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. pages 3-10. See Figure 4, and page 6.	1-7,12-14, 17,19
Y	de Serres et al., "Chemial Mutagens. Principles and methods for their detection", published 1980, Plenum Press, New York. pages 331, and 365-473. See at least pages 331, 367-369, 377.	1-7,12-19
Y	Science Volume 227, issued 15 March 1985, (Engebrecht et al.) "Measuring gene expression with light". pages 1345-1347. See entire document.	1-7,12-19
Y	Science Volume 236, issued 05 June 1987, (Maniatis et al.) "Regulation of inducible tissue-specific gene expression. pages 1237-1245 See pages 1237, 1239, 1240, 1243.	1-7,12-19
Y	Molec. Cell. Biol. Volume 7, No. 2, issued February 1987, (de Wet et al.) "Firefly luciferase gene: Structure and expression in mammalian cells'. pages 725-737. See the abstract, Fig. 1 and 3, pages 729-734.	1-7, 12-19

Attachment to Form PCT/ISA/210

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Search Terms:

mammal, toxicity, toxic, chemial, chemicals, carcinogen, carcinogenic, carcinogen, oncogenic, oncogen, transcript