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<p>(54) Title: THE L-PLASTIN PROMOTER REGION AND ITS USES</p>		
<p>(57) Abstract</p> <p>The L-plastin gene promoter and regulatory region is provided for use as a transcriptional and translational vector of other genes for expression in mammalian hosts. The L-plastin promoter with a limited portion of the regulatory region functions as a strong promoter. The promoter together with the upstream regulatory region is active specifically in hemopoietic cells and in transformed cells of solid tissues which are of non-hemopoietic origin and is inducible in response to estrogen and progesterone in hormone responsive cells.</p>		

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THE L-PLASTIN PROMOTER REGION AND ITS USES

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BACKGROUND OF THE INVENTIONField of the Invention

This invention relates to the nucleotide sequences corresponding to the L-plastin promoter region and their uses.

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Description of the Prior Art

Plastin was first noted as a polypeptide which appeared to be induced abundantly accompanying tumorigenic transformation of human cells. Plastins are a family of highly conserved actin-binding proteins approximately 70 kd in size. In yeast, a plastin homolog was found to be required for actin organization and morphogenesis.

In humans and rodents, there are two ubiquitous plastin isoforms: the L isoform expressed in cells of hemopoietic lineages and the T isoform expressed in the other cells of solid tissues. These two isoforms are highly related in amino acid sequence (80% homology) but are encoded by two separate single copy genes. Regulation of the tissue-specific constitutive expression of these two genes appears to be highly stringent because only one isoform has been found in each normal cell type thus far examined.

Such stringent control of plastin gene expression is, however, lost accompanying neoplastic transformation as many different types of human solid tumor-derived cell lines express L-plastin in addition to T-plastin, indicating that the L-plastin gene is constitutively activated in these cells at the time of transformation. In a survey of 50 human tumor cell lines and primary tumor cell strains derived from non-hemopoietic stem cell types, 65% expressed L-plastin. The highest correlation of L-plastin expression was among epithelial tumors, more specifically tumors of certain female reproductive organs (mammary, ovarian, and chorio carcinomas).

SV40-mediated transformation can also lead to activation of the L-plastin gene as it has been shown that SV40-transformed MRC fibroblasts express L-plastin while the parental normal MRC fibroblasts do not express L-plastin.

5 Since L-plastin expression is a frequent event in human solid tumor formation, understanding the mechanism by which the L-plastin gene is activated may shed light on a fundamental aspect of human tumorigenesis.

10

SUMMARY OF THE INVENTION

The L-plastin gene promoter and regulatory region is provided for use as a transcriptional and translational vector of other genes for expression in mammalian hosts. The L-plastin promoter with a limited portion of the regulatory
15 region functions as a strong promoter. The promoter together with the upstream regulatory region is active in hemopoietic cells and in transformed cells of solid tissues which are of non-hemopoietic origin and is inducible in response to estrogen and progesterone.

20

In particular, the invention comprises a nucleotide sequence of not more than about 5.0 kilobases comprising a nucleotide sequence corresponding to the sequence of the L-plastin promoter, preferably including at least a portion of the upstream regulatory region. An expression construct
25 comprising a nucleotide sequence corresponding to the sequence of the L-plastin promoter and a foreign gene is also provided. The construct preferably includes sequences corresponding to at least about two kilobases of the upstream regulatory sequences, more preferably about five kilobases of the
30 upstream regulatory sequences.

The invention also provides a method for inducing steroid-responsive production of RNA in a cell which is estrogen-responsive or progesterone-responsive comprising engineering the cell with an expression vector comprising a
35 nucleotide sequence coding for said RNA sequence and a nucleotide sequence corresponding to the L-plastin promoter and a portion of the upstream regulatory region containing a

progesterone responsive element or the estrogen responsive element. The nucleotide sequence is preferably a DNA sequence encoding a protein. The invention provides a method for production of RNA in a hemopoietic cell comprising engineering
5 said cell with an expression vector of this invention.

In a preferred embodiment, the invention comprises a method for determining whether an agent is cancer-causing. The method comprises contacting a non-malignant tissue cell engineered with an expression vector comprising the L-plastin
10 promoter and a reporter gene with said agent and observing the cell for the expression of the reporter gene, the expression of said reporter gene indicating that the agent is cancer causing. In addition, an expression vector of this invention can be used to express a protein only in cancer cells which
15 are present in a mixed population of cancer cells and normal tissue cells.

DETAILED DESCRIPTION OF THE INVENTION

The L-plastin promoter and regulatory region, expression
20 constructs containing the L-plastin promoter (or the L-plastin promoter and regulatory region) and methods employing the L-plastin promoter are provided for the expression of RNA and polypeptides in mammalian cells, in particular hemopoietic cells and cancer cells of hemopoietic and non-hemopoietic
25 origin. The sequences can be employed for expression of RNA and L-plastin or other polypeptides, usually other polypeptides. The sequences are not active in cells which do not express L-plastin. In particular, the sequences are not active in a normal tissue cell, but are active when the cell
30 is transformed, if the endogenous L-plastin gene has been activated by the transforming event.

Therefore, when the constructs include a reporter gene, the constructs can be used to identify transformed cells and to identify agents that induce transformation. In addition,
35 the L-plastin regulatory region contains an active estrogen receptor element and one or two active progesterone receptor elements. Therefore, the L-plastin promoter and a portion of

the regulatory region can be used to provide hormonally regulated expression of a protein (or production of RNA), in a cell with the appropriate receptors.

5 DESCRIPTION OF SEQUENCE

The sequence of the L-plastin promoter and upstream regulatory region is described below. The term "the L-plastin promoter region" as used herein, refers to the L-plastin promoter and at least a portion of the upstream regulatory region. The L-plastin promoter and its regulation in normal and transformed cells was characterized. Genomic DNA spanning the promoter region of the gene was sequenced. Table 1 illustrates genomic fragments of the promoter and gene region obtained by use of various restriction enzymes. The nucleotide numbers in the table refer to the location of the restriction sites with 1 being the transcription initiation site nearest the TATAAA box. The length of the promoter region does not include coding region sequences that may be present in the fragment.

20

TABLE 1

Fragment No.	Restriction Sites	Nucleotide Nos.	Length of Promoter Region
1	PvuII/ScaI	-204 to +111	0.3 kb
5 2	HincII/ScaI	-500 to +111	0.6 kb
3	ScaI/ScaI	-2265 to +111	2.4 kb
4	PvuII/HindIII	-204 to +746	0.9 kb
5	EcoRI/HindIII	-4354 to +746	5.1 kb

10 The sequence of the promoter and the sequenced portion of the upstream flanking region are shown in Table 2. Specifically, the L-plastin promoter and regulatory region was an approximately 5.1 kb region upstream from the promoter from the EcoRI site to the HindIII site (Fragment No. 5 in Table 15 1). The first approximately 2.1 kb of the region from the promoter upstream was sequenced and that sequence is shown in Table 2. Throughout the application, the nucleotide numbers refer to the numbering system used in Table 2. In Table 2, nucleotide 1 represents the transcription initiation site 20 closest to the TATAAA box. The TATAAA box (bases -15 to -10) is underlined. The 3' end of the first exon (nucleotide 140) is indicated by an asterisk (*). Upstream sequences homologous to various transcription factor binding motifs are underlined and indicated as follows: *Ets-1*; *PR*, progesterone responsive 25 element; *ER*, estrogen responsive element; *AP1*; and *SP1*.

TABLE 2

-2265	AGTACTATGC TGCACAAGCA ATTATAAACA CCAACAGCAA AAAAATACAC TTCTCTGAAA	60
-2205	AAGTCTTGGT CTAGGACCTA AACAAATTGCC TGAAACTGGG TAGACTTACA CCAATGAGAG	120
-2145	GCAGATAAAG AGATTAAGAT TGAGGGAGTA GGGCAGGGCT TGCAATGGTG CCGGCCAGGA	180
-2085	TGTGGCTGAG GGGGTGTGGG TGCCTGCCGT GGATGCTAGG GTAGAAGACG ACTCTATATA	240
-2025	CTGGGTGGCT GTAAGCAGTA CCCAGGTCAA TGCCTTTTCAT CTTCTACAAC CTCGACGGTTG	300
-1965	CCTGGAATCC TAAATCTTTT TCTTCACTTA ACAAACATCA CCTCTGCTCA AATCTGCAAC	360
-1905	TGCTTTGATA TCACACTGCC TTTTTCACCC CTCTATTATA GATGGCATTT ATTACTTAC	420
-1845	ATGTTTTTTC CCCACTAGAC TATACTCCTT GAGAACAGCG ATTGTGTCTT ATTTATTCT	480
-1785	GAATCACCAA TTCAGACAGG CATGCAAACA CTTGCTGAAC CAATGCCAAA ATATATTTTG	540
-1725	CTCTTCTTCA TAGATTCTC CGGCCTCAGA TGACCAGGCA CCACTAGATA CAGAACA <u>CTG</u>	600
-1665	TGCTTTCTCTT CTCCAAGGTA AAGGAATAAA TATCTGTTC CCTTCATGAA GTGTTACTGT	660
-1605	TGGGCCTTTA TGCCATCCTG AAGCCACCAG <u>GATGTGGAAC</u> CAGATCAGGG AGGTCCACAG	720

Ets-1

5

Ets-1

1
6
1

15

PR

-1545 TTACAACCCC TTGTATCTGT AACACCAGCA GGACATTATC TACAGAGTGG TGCTGCAGGG 780

-1485 CCCCGAATGA AGACAGCATT TTGCTGCTTT GTAGCGGTGAG CAGTGTCTGTA ACAGTGATGC 840

5 -1425 ATGGATGTTT CTCTGGTGTG CTGAAAGAAT GTAGGTGCTT CTTGAAAGCT CTCTGCAACT 900

-1365 TATTAATTGG GAGTGATTAT GCGATGGAGA AAACAGAGTC CCCATCACCC CCTCAGTCTT 960

10 -1305 CCTGGGAAA TCACAAGAGG GCTGATAGCT CTCTGTGAGG TGAACCGTTT CTAGAATCCC 1020

-1245 CACCGTCTCG TCCTGTCTTT CCGCCCCACCC AGTTCCTCAA GATAGCCCCCT GTGGGCTTCT 1080

-1185 GATGAAGTCA CCACACCACT GGCTAATGAA GTAGATAAAC CAGAACAGTT TGGTTAACA 1140

15 -1125 TTTAAGGTCA GAAACAGGAA CTTTCTAGAG GAGAAATCAA AAAAGCAAAA GAAGTATAAG 1200

-1065 GGCAGCCCTC CAACCAGTCA GAATACCGTG ACCACCTGAG AGGCCCGTGG CCCAGCGGAC 1260

20 -1005 ACGGACGCAT GTCAACTCTG GAGCAGATAT CTTCAGCGCA GCATCTGACC TGGGAGTACA 1320

-945 GCCACATACC CTCATTCCCTA AACGGCAGAT TGACTIONTGG AGTCACACAC AGTCTCCGGG 1380

-885 CAATGTGGAG ACATGTCTAA TATTTAGTCA ACATAACTCA GGGTGCCACA GTCTTCACAA 1440

PR

Ets-1

-825 CTGTTGTGAG CACTTGAGGA TGCTCCATTT GAAGATAGGA ATTTGCCCTC AAGCATCTGG 1500

-765 GGTTGGGTA CAGAACAGAG CTTCCTCTGC CACCACCTGC TAATTTTATA AATGTGCATT 1560

-705 CAAAAAAAAA TCCTGCCCTGT AAGAAGGAAT TAAGCTACCC ATTTAAATAT AACAGCTGCC 1620

-645 TGTGCAATCT ACTGCTGCTC TTTATAGGAA ACGCTTAAAT AATGAGATA CTTAATTTGGG 1680

10 -585 TTAAAAGAGAT CCCTAGCACA TAGATGTTCT ATAAATAAAA GAATGAGTAA ATAATCTAGT 1740

AP1

-525 AACCTTCCTT TTCATGTCCT TCACTTAAAG AGATCGTTCT GTTTTGTTTG CACCAATAAG 1800

-465 ATCACTGTTA GAGGACTCCA GAGAGTTTG ATTTCAGGTG GGGTGGGGCT TTCCCAAGGA 1860

15 -405 AGTCCCTTTT CATTGTTTCA GGTGFACTGC CACCTTTTTC CCTGGCTCTT TCACTAAAAA 1920

-345 TGA AAAAATTT GTTGATCTTT GCTGTAAGTA GGTAGGCATC TGGGCTTTGC TTTTGCAACT 1980

20 -285 AGAGTCAAAG AAGTCAAGTT ATCAGGCTGA TCTTGCCTTG CTATCTAGAA TCAGAAAGGT 2040

-225 TTAAGTAGCC CAGGGACTAC TCAAAGACAG CTGGAGGAGA AAGGGAGAGA GAAAAATGCT 2100

556 GGGTAGTTTA ATTTTAGTTC TCGGGTCTG TTTTCTAAA AAACCTCTTT ACAGTACTTT 2880
 616 AGTGGATAAA TTATCTAGAG TCATATGTTA CTGAATGTGA TACTGGTTAT TTGAGTGATT 2940
 5 676 TTGGGGGCCA AGGGTTTTTA ATGGGTGTGC TTTATAACCA CATGGAATTA GAGGTTTTCT 3000
 736 CTTTCCATTT GTTCTCTAAA AATAAAAGCA ATTTGTATAA TGCTTAATTG TAGTGTTTTT 3060
 796 GTAGGATTGT CTGAATTATT TGTTATGCTT ATGACCATAT CAATGAATAT TCACTCAAAT 3120
 10 856 TCTGGGAGAA AGGCTTTTAT CTACAATAGA AATTTTACA CAGTTGATAT GGGATATTTA 3180
 916 TTTCAGAAAC ATGCCCAAAC ATAGACACTT TGGATTTTGT AGTATAAGCT TCCATAAATG 3240
 15 976 CACT 3244

As shown in Table 2, the promoter region contained multiple transcription start sites which were mapped using standard primer extension and S1 nuclease mapping methods. In addition, several potential cis-acting regulatory elements were identified flanking the TATAAA box. The L-plastin promoter was flanked by progesterone and estrogen responsive elements. This finding was surprising since this gene encodes a ubiquitous and abundant hemopoietic cell architectural protein.

10 The promoter and regulatory elements of the L-plastin gene were characterized. Transcription initiation from this promoter was found to occur at multiple sites and as near as 10 bp from the 3' side of the TATAAA box. The promoter and its flanking DNA was cloned and sequenced to identify
15 potential regulatory elements that participate in the induction of the L-plastin gene in neoplastic cells. Examination of upstream sequences revealed the existence of two progesterone, one estrogen, and four Ets-1 responsive elements flanking the promoter.

20 A 315 bp fragment spanning the TATAAA box, an Ets-1 binding site, an estrogen responsive element, and an Sp1 binding site exhibited maximum promoter activity using CAT (chloramphenicol acetyltransferase) as a reporter while longer promoter fragments extending into upstream flanking sequences
25 spanning the two progesterone responsive elements AP1 site and 3 potential Ets-1 sites exhibited reduced promoter activity.

Although the L-plastin gene promoter has a classic TATAAA box, which usually directs transcription initiation at a single site about 30 bp downstream, transcription initiation
30 occurs at multiple sites. In addition, transcription initiation can occur as close as 10 bp from the TATAAA box. Nevertheless, the presence of a perfect TATAAA box and an adjacent Sp1 binding site in the L-plastin promoter indicates its potency in promoting highly efficient transcription, as
35 demonstrated in the Examples.

PROMOTER AND TRANSCRIPTION SYSTEM

As described in detail below and in the Examples, the L-plastin promoter region contains upstream regulatory sequences that both inhibit the activity of the promoter and contain regulatory elements that make the activity responsive to estrogen and/or progesterone.

For use of a transcription initiation system of this invention as a strong promoter, the L-plastin promoter region sequence spanning the TATAAA box the Sp1 site, and the proximal Ets -1 site is preferred (fragments 1 and 4 in Table 1 contain these sequences). Conveniently, the region from the PvuII site through the ScaI site (Fragment No. 1 in Table 1), which is about 315 nt is used. This portion of the L-plastin promoter region which functions as a strong promoter can be used for expression in hemopoietic cells or non-hemopoietic cell.

For the sequences of this invention, use of sequences corresponding to the native sequence is also contemplated. The phrase "corresponding thereto" means that the sequences can contain nucleotides that are not identical to those of the L-plastin promoter region sequence. Those non-identical sequences can be substitutions in the sequence, insertions or deletions. Numerous changes can be made in the native sequence that preserve the ability of the corresponding sequence to be active as a promoter and be regulated by the desired cis acting elements. For example, a modified sequence in which the estrogen responsive element or one or both progesterone responsive elements has been deleted or mutated may be preferable.

In addition, so long as key regions such as the TATAAA box and the initiation site are preserved, changes in the sequence can be made and preserve the activity of the sequence as a promoter. Preferably, any alterations preserve the activity of the promoter and the regulatory elements. Most preferably, the sequences are identical to that of the native promoter with any engineered changes in the promoter that are desired, such as deletion of a selected regulatory element.

For use of a transcription initiation system of this invention for expression of genes in cancer cells of solid tissues or for expression of genes limited to hemopoietic cells, a sequence corresponding to the sequence from the 5 transcription initiation region through the proximal Ets-1 site (-111), more preferably through the distal Ets-1 site, most preferably at least about the first two kilobases (kb) of the L-plastin promoter region is used. Conveniently, about a five kilobase region from the HindIII site to the EcoRI site 10 (Fragment No. 5 in Table 1) is used, since the region has been experimentally shown to be active only in cells which express the endogenous L-plastin gene.

To express a protein or RNA sequence in response to steroids, the region extending from the first transcription 15 initiation site through the first progesterone responsive element, preferably through the second progesterone responsive element can be used for hormone responsive expression of a structural gene in a cell with an estrogen and a progesterone receptor. To express a structural gene in response to only 20 estrogen or progesterone, but not both, the L-plastin promoter region can be used in a cell with only the estrogen or the progesterone receptor.

To produce an L-plastin promoter region responsive to estrogen but not progesterone, the sequences corresponding to 25 the regulatory region should extend through the estrogen responsive element but not extend through the proximal progesterone responsive element.

To produce an L-plastin promoter region responsive to progesterone but not to estrogen, the sequences corresponding 30 to the estrogen responsive element should be mutated so that the estrogen responsive element is no longer active. For example, a region starting at a restriction site upstream from the estrogen responsive element through a downstream restriction site can be amplified using a primer that starts 35 at the closest restriction site and spans the estrogen responsive element. The primer is sufficiently homologous to bind to and amplify the region, but contains sufficient

mismatches to ensure that the amplified region contains an inactive estrogen responsive element. Following amplification, the mutated amplified sequence can be ligated into the remainder of the promoter region sequence so that the modified sequence is identical to the native sequence except at the selected sites in the mutated estrogen responsive element region.

In addition, use of the portion of the L-plastin promoter region through the estrogen responsive element for estrogen-dependent expression in a cell with estrogen receptors is contemplated. For progesterone-dependent expression, the region of the L-plastin promoter region surrounding the estrogen responsive element can be removed or mutated so that the region is not functional. Techniques for selectively mutating or excising a portion of a nucleotide sequence are well known.

For example, to produce an L-plastin promoter region responsive to estrogen but not progesterone, the sequences corresponding to the regulatory region should extend through the estrogen responsive element but not extend through the proximal progesterone responsive element. To produce an L-plastin promoter region responsive to progesterone but not to estrogen, the sequences corresponding to the estrogen responsive element should be mutated so that the estrogen responsive element is no longer active. For example, the region a restriction site upstream from the estrogen responsive element through a downstream restriction site can be amplified using a primer that starts at the closest restriction site and spans the estrogen responsive element. The primer is sufficiently homologous to bind to and amplify the region, but contains sufficient mismatches to ensure that the amplified region contains an inactive estrogen responsive element. Following amplification, the mutated amplified sequence can be ligated into the remainder of the promoter region sequence so that the modified sequence is identical to the native sequence except at the selected sites in the mutated estrogen responsive element region.

Experiments demonstrating the multiple initiation sites as well as the strength of the promoter are described in detail in the Examples. The strength of this promoter was demonstrated using truncated genomic fragments. Fragments 5 which included the regulatory region sequences 180 bp upstream from the TATAAA box showed the strongest promoter activity, which was nearly equal to the activity of the β -actin promoter, a well known strong promoter. However, this activity of the promoter alters as upstream negative control 10 elements affect its activity. Specifically, when the upstream elements to the EcoRI site (approximately 5.1 kb from the promoter) are present, transcription and translation of the gene are inhibited. As described in the Examples, cell lines that did not exhibit expression of the endogenous L-plastin 15 gene exhibited a low frequency of transfectant colonies which activated the recombinant β -galactosidase under control of the L-plastin promoter. Such colonies, which appeared at low frequency, were shown to have activated the endogenous L-plastin gene as described in the Examples.

20

EXPRESSION VECTOR

An expression vector of this invention comprises the L-plastin promoter transcription initiation region together with either L-plastin or a foreign gene, usually a foreign 25 gene. As is well known, a transcription initiation region can be used to express a protein or produce RNA (as for production of antisense sequences) in a mammalian cell. The regulatory region can be added to provide the described regulation. The portions of the L-plastin promoter region used for production 30 of RNA in various types of cells and under various types of regulation have been described previously.

The expression of L-plastin or a foreign protein may be achieved in a variety of ways in mammalian host cells. The expression construct involves the L-plastin promoter region 35 and the structural gene present as a contiguous entity or as exons separated by one or more introns. The expression construct may be joined to an appropriate vector, if desired.

By a vector is intended a replication system utilized by the intended host. Usually the expression construct includes one or more markers to ensure the stable maintenance of the DNA construct in the host. Preferably the construct contains a 5 marker gene to determine presence of the construct in the cell and a reporter gene to monitor promoter activity.

Various replication systems include bacterial and viral replication systems, such as retroviruses, simian virus, bovine papilloma virus, or the like. Alternatively, one may 10 combine the DNA construct with a gene which allows for selection in a host. This gene can complement an auxotrophic host or provide protection from a biocide. Illustrative genes include thymidine kinase, dihydrofolate reductase, which provides protection from methotrexate, or the like. For 15 example, markers can provide resistance to a biocide, e.g., G418, methotrexate, etc.; resistance to a heavy metal, e.g., copper; prototrophy to an auxotroph; or the like. Suitable genes for selection of a host cell include thymidine kinase, dihydrofolate reductase, metallothionein, and the like. 20 Alternatively, marker genes can express a detectable protein to determine activation of the promoter in a host. Reporter genes are well known and include CAT and, preferably, β -galactosidase.

In addition, the subject gene or antisense sequence to be 25 expressed may be joined to an amplifiable gene, so that multiple copies of the sequence of interest may be made. Depending upon the particular system, the gene may be maintained on an extrachromosomal element or be integrated into the host genome.

30 The foreign gene may come from a wide variety of sources such as prokaryotes, eukaryotes, pathogens, fungi, plants, mammals, including primates, particularly humans, or the like. These proteins may include hormones, lymphokines, enzymes, capsid proteins, membrane proteins, structural proteins, 35 growth factors and inhibitors, blood proteins, immunoglobulins, etc.

Of particular interest are proteins to be expressed in cells which are responsive to estrogen or progesterone, in hemopoietic cells or cancer cells. The constructs can be used to produce therapeutic proteins, viral resistance proteins, 5 and proteins involved in repair of genetic defects. In addition, the constructs can be used to produce antisense RNA and antisense ribozymes.

The manner in which an individual DNA sequence coding for a protein or antisense sequence of interest is obtained, 10 divided into individual exons, and joined to the transcriptional and translational regulatory signals of the L-plastin gene will depend upon each individual polypeptide of interest, as well as the information available concerning the DNA sequence coding for such polypeptide.

15 The L-plastin promoter or transcription system including the promoter may be used for the regulation of expression of other genes by regulating transcription of mRNA complementary to another mRNA or portion thereof. In effect, the L-plastin promoter would regulate transcription of the nonsense strand 20 or portion thereof of the gene whose expression is to be inhibited. Such inhibition may find use in making an auxotrophic host, inhibiting one pathway in favor of another metabolic pathway, reversing or enhancing oncogenic characteristics of a cell, or the like.

25 Introduction of the DNA into the host will vary depending upon the particular construction. Introduction can be achieved by any suitable gene transfer technique such as transfection, transformation, transduction, or the like, as amply described in scientific literature. For production of 30 large amounts of proteins or antisense sequences, the host cells will normally be immortalized cells, that is, cells that can be continuously passaged in culture.

For the most part, these cells will be normal and may be any convenient mammalian cell, which is able to express the 35 desired polypeptide, and where necessary or desirable, process the polypeptide, so as to provide a mature polypeptide. Processing the polypeptide can include glycosylation,

methylation, terminal acylation, e.g., formylation or acetylation, cleavage, or the like. In some instances it may be desirable to provide a leader sequence providing for secretion or directing the product to a particular locus in the cell. For secretion, the host should be able to recognize the leader sequence and the processing signal for peptidase cleavage and removal of the leader.

In addition to use for expression of a protein, as in a cell culture where the protein is harvested and used, the constructs can be used for expression of proteins in fertilized eggs for development of transgenic animals or in human cells removed from the body, engineered and put back into the body (e.g.; lymphocytes). A discussion of use of the L-plastin promoter region in hemopoietic cells and for steroid regulated expression is found hereinafter.

GENETIC ENGINEERING OF ESTROGEN AND/OR PROGESTERONE RESPONSIVE CELLS AND HEMOPOIETIC CELLS

In addition to using the L-plastin promoter region as a transcription system to express a foreign protein, the L-plastin promoter finds particular application in genetic engineering of hemopoietic cells where constitutive expression is desired and in tissue cells where gene expression in response to progesterone or estrogen is desired.

As stated previously, portion of the L-plastin promoter region can be used so that the L-plastin promoter region is active only in hemopoietic cells. The promoter can be present in an expression vector for expression limited to hemopoietic cells. In this way, one can engineer expression of a protein (or production of an antisense sequence) only in the hemopoietic cells of a mixed population of cells.

Alternatively, the L-plastin promoter region can be used for expression of proteins in lymphocytes. The protein included in the construct is expressed at high levels, similar to the level of expression of L-plastin in hemopoietic cells. For example, the engineered protein or RNA can be used for HIV therapy in lymphocytes.

For genetic engineering of non-hemopoietic cells for expression of the protein (or production of an RNA sequence) in response to progesterone and/or estrogen, the selected coding region is placed under the transcriptional control of
5 the portion of the L-plastin promoter region described previously. The engineered cells are then subject to hormonal control. The cells can be used for expression of a selected protein in response to the environment in female reproductive tissues. For example, a protein which is genetically
10 defective and leads to loss of pregnancy can be engineered into the cells and expressed in response to the changing hormonal levels during pregnancy. In addition, engineered cells facilitate study of the levels of expression of various proteins during the course of pregnancy.

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GENETIC ENGINEERING OF TISSUE CELLS FOR EVALUATION OF
CARCINOGENIC AGENTS

As stated previously and demonstrated in the Examples, the L-plastin gene is normally expressed only in hemopoietic
20 cells. However, the gene is activated in other cell types of solid tissues accompanying tumorigenesis. Engineered cells containing an expression vector of this invention can be used to evaluate whether an agent or condition is cancer causing. For this purpose, the expression vector will contain a
25 reporter gene in addition to the L-plastin promoter. Suitable reporter genes are well known and were described previously. In a preferred embodiment, the reporter gene is the E. coli β -galactosidase gene or neomycin resistance gene (neo) which confers resistance to the drug G418.

30 The Examples demonstrate that the 5.1 kb promoter fragment of the L-plastin gene contains control elements which participate in the suppression of its activity in normal cells and its activation in human tumor cells. The demonstrated ability of the β -galactosidase assay to discriminate between
35 normal and neoplastic cells that do not have an active endogenous L-plastin gene and the tumor cells that exhibit activated expression of L-plastin demonstrates that cells

engineered with an expression vector of this invention can be used for early detection of nascent in vitro neoplastic cells that also have activated expression of the endogenous the L-plastin gene.

5 By putting β -galactosidase or other reporter genes under the control of the 5.1 kb L-plastin promoter fragment, a reporter gene can be inserted into normal cells which remains silent until transformation by a mechanism leading to activation of the endogenous L-plastin gene. The reporter
10 gene is activated at the time of some transformation events which catalyze activation of the endogenous L-plastin gene and expression of the reporter gene signals the onset of the development of the neoplastic or tumorigenic state.

In addition, growth selectable markers like neomycin-
15 resistance (G418-resistance) can be put under the control of this promoter to select nascent neoplastic cells from a normal cell population within several population doublings after transformation which is accompanied by activation of the endogenous L-plastin gene. Such a selection system allows
20 early selection of transformed cells in vitro. These applications provide a new experimental approach to studying the neoplastic transformation in diploid human cells which are difficult to transform in vitro. Since nascent cancer cells may be selectively inhibited in growth in vitro compared to
25 normal nontransformed cells, use of a growth selectable marker such as G418-resistance under the control of the L-plastin promoter will facilitate identification, isolation, and propagation of the nascent cancer cell for further evaluation and characterization.

30

USE OF THE RECOMBINANT L-PLASTIN PROMOTER TO DIRECT SYNTHESIS OF A TOXIN TO CANCER CELLS AND UNDESIRABLE HEMOPOIETIC CELLS

As discussed previously, the L-plastin promoter is specifically and constitutively active in many cancer cell
35 types and in normal leukocytes. Therefore, a recombinant gene comprising a gene encoding a cytotoxic product (hereinafter "toxin gene") under the control of the L-plastin promoter is

specifically active in these target cell types and can be used to kill these cell types specifically. Cells that can be targeted by a recombinant toxin gene of this invention include any cancer cell in which the L-plastin promoter is active.

5 Leukocytes that are neoplastic or infected with a pathogenic virus such as HIV or HTLV are examples of hemopoietic cells that are suitable targets for a recombinant toxin gene of this invention. Undesirable leukocytes or other selected cells can be targeted using a ligand that binds to a receptor that is
10 specific for the intended target cell type.

The encoded cytotoxic product can be a toxin which generally kills cells such as diphtheria toxin or ricin or a toxin that kills either cancer cells or leukocytes specifically. The choice of the encoded toxin for a
15 particular target cell population depends on the sensitivity of the selected target cells to the toxin. More specifically, some toxins kill certain cancer cells but do not kill leukocytes, and vice versa. For example, certain cytokines, such as tumor necrosis factor and interferon, have inhibitory
20 effects on the growth of cancer cells. These inhibitory effects are not apparent on normal eukaryotic cells, including leukocytes. In a preferred embodiment, the gene for the encoded toxin of choice that is placed under the control of the L-plastin promoter encodes a toxin that provides
25 specificity toward killing only the intended target cell type.

A method for producing the toxin in cancer cells and/or leukocytes comprises transfecting these eukaryotic cells with a vector encoding a toxin gene, the toxin gene being under the control of an L-plastin promoter. The recombinant toxin gene
30 can be used to transfect a mixed population of cells which population includes both normal, non-target cells and target cells where it is desired that the recombinant toxin gene is expressed selectively in the target cells under the control of the L-plastin promoter. Such mixed populations include normal
35 tissue that contains cancerous cells.

In another embodiment, general toxins like diphtheria toxin or ricin whose synthesis is placed under the control of

the L-plastin promoter can be specifically directed to the intended target cell through the use of drug delivery systems. Such drug delivery systems include use of liposomes or retroviruses having an antibody combining site, a receptor, or
5 the like which directs the liposome or retrovirus encapsulated drug to the target cells. These drug delivery systems are well known and do not constitute part of this invention.

In addition, general toxins can be genetically modified to kill only the cell in which they are synthesized, thus
10 preventing the spread of the toxin from the intended target cell to surrounding cells that are not the intended targets. For example, diphtheria toxin mutants have been developed in vitro that cannot enter eukaryotic cells but retain toxic activity if synthesized inside the target cells.

15 Example 8 illustrates use of the L-plastin promoter to express a foreign gene (neo) in transformed cells transfected with a construct comprising the promoter and the foreign gene. This example demonstrates that foreign genes can be expressed using the L-plastin promoter in cancer cells which
20 endogenously express L-plastin. Example 7 demonstrates that most, if not all neoplastic human cells exhibit some degree of activation of the L-plastin gene. In addition, the example study demonstrates that a foreign gene, such as neo, attached to the L-plastin promoter was expressed in cells having
25 endogenous activation of the L-plastin gene.

USE OF THE RECOMBINANT L-PLASTIN PROMOTER TO DIRECT SYNTHESIS
OF A SELECTABLE MARKER SPECIFICALLY IN TARGET CANCER CELLS AND
HEMOPOIETIC CELLS FOR RECOVERY OF THESE INTENDED TARGET CELLS
30 FROM THE BODY AFTER GENE THERAPY

In some cases, it may be useful or necessary to recover the cells that are the intended targets for gene therapy as described above. For example, recovery of these cells facilitates the characterization of properties of the cells
35 such as chromosomal ploidy, cellular protein synthesis, and oncogene activation. In addition, recovery of target cells allows monitoring of the efficacy and safety of L-plastin

therapy. This recovery can be accomplished by in vivo or ex vivo delivery of a recombinant drug resistance gene under the control of the L-plastin promoter.

A method for recovering the intended target cells
5 comprises transfecting a population of cells containing the target cells with a vector encoding a selectable gene that is under the control of the L-plastin promoter. The selectable gene can be any suitable drug resistance gene, i.e., any gene that encodes a protein which confers resistance to a drug,
10 such as the antibiotic neomycin (G418 analogue). The cell population can be transfected either in vivo or ex vivo. Following delivery of the selectable gene to the intended target cells, a transfected target cell can be selectively cultured and replicated in vitro from the tissue biopsy or
15 body fluids (blood, mucus, urine amniotic fluid, etc.) because the transfected cell is capable of activating the L-plastin promoter.

Using this method the target cell can be isolated in the presence of an overwhelming majority of other cells which
20 either lack an active L-plastin gene, lack the ability to activate the recombinant L-plastin promoter, or have not been targeted by the drug delivery system to receive the recombinant selectable gene under the control of the L-plastin promoter. Growth of the intended target cell out of the
25 biopsy or body fluid requires culturing of those cells under selective conditions. When the selectable gene is a drug resistance gene, the cells are cultured in the presence of a suitable concentration of the appropriate drug such that the untransfected cells or transfected cells lacking the ability
30 to activate the recombinant L-plastin promoter are killed by the drug or are unable to replicate in the presence of the drug, while the cells that synthesize the product of the drug resistance gene are not killed and can replicate in the presence of the drug. After a sufficient culturing time, all
35 untransfected cells will be killed. The remaining replicating cells in the culture are the target cells. These cells can then be examined to determine their relevant properties.

This invention is further illustrated by the following specific but non-limiting examples. Temperatures are given in degrees Centigrade and concentrations as weight percent unless otherwise specified. Procedures which are constructively reduced to practice are described in the present tense, and procedures which have been carried out in the laboratory are set forth in the past tense.

EXAMPLE 1

10 *Isolation and Characterization of
 the L-plastin Promoter Region*

The L-plastin promoter was isolated and characterized using the following procedures.

15 **Cell cultures.** The cell cultures used were cell lines HuT-12, HuT-14, HOS, HT1080, MG63, RD, Wi-38VA13, Wi-26VA4, and rat-2. Those cell lines have been described in the literature and are available commercially from Leavitt et al., *Mol. Cell Biol.* 7:2457 (1987). All cell types were cultured
20 in MEM- α medium (Sigma) supplemented with 10% fetal calf serum and antibiotics.

CAT plasmid construction and CAT assay. L-plastin genomic DNA fragments attached with XmaI linkers were cloned into the XmaI site of PUMSVOCAT, which was described by Salier
25 and Kurachi *Biotechniques*, 7:30 (1989). To serve as a positive control for CAT assay, a 4.3 kb EcoRI-HindIII fragment containing the β -actin promoter was derived from the plasmid Ph β Apr-1-neo Gunning et al., *Proc. Nat'l Acad. Science USA* 84:4831 (1987) and cloned into PUMSVOCAT. Transfection of
30 plasmid DNA into HuT-14 cells was performed by the calcium phosphate precipitation method Ng et al., *Nucleic Acids Res.* 17:601 (1989). Cells grown in a 100 mm dish were harvested at 80-90% confluency, centrifuged, resuspended in 200 ml PBS, and lysed by freeze-thaw.

35 After removing the insoluble cell debris by centrifugation, each cell lysate was measured for protein concentration by the protein assay kit of Bio-Rad. CAT assay

was then performed with the CAT ELISA kit purchased from 5 Prime--3 Prime, Inc. (West Chester, P.A.).

Primer extension. A 25-mer oligonucleotide complimentary to the mRNA and corresponding to the end of the first exon 5 (bases 125 to 149; see Table 2) was labeled at the 5' end with $\gamma^{32}\text{P}$ -ATP. After labeling, the oligonucleotide (0.1 μg) was precipitated with ethanol and resuspended in 20 μl of distilled water. A 10X dilution was made 1 μl of which was annealed to 16 μg of cellular RNA in a 10 μl solution 10 containing 250 mM KCl and 10 mM Tris, pH 8.3. Annealing was done by incubating the solution at 80°C for 3 minutes and then at 56°C for 1 hour. The annealed oligonucleotide was then extended by the addition of 24 μl of a mixture containing 40 mM Tris, pH 8.3, 15 mM MgCl_2 , 5 mM DTT, 1.8 μg actinomycin, 15 1.2 mM of each of dATP, dCTP, dGTP, and TTP, 35 units of human placental RNAase inhibitor, and 100 units of SuperScript reverse transcriptase (BRL).

After 45 minutes of incubation at 45°C, the reaction was terminated by the addition of 200 μl of 0.5% SDS, 200 mM NaCl, 20 and 20 mM EDTA. The mixture was extracted with phenol/chloroform and precipitated with ethanol. The pellet was resuspended in sequencing dye solution plus 5 mM of NaOH, heated at 90°C for 10 minutes, and loaded onto an 8% polyacrylamide sequencing gel. After electrophoresis, the gel 25 was dried and exposed to an X-ray film.

Preparation of single-stranded probe. The same $\gamma^{32}\text{P}$ -labeled 25-mer used in the primer extension experiments was annealed to a single-stranded M13 DNA that contained a portion of the L-plastin genomic DNA (bases -257 to 966; see 30 Table 2). The annealing mixture was composed of 48 μl (4.8 μg) of M13 DNA, 16 μl of annealing buffer (0.1 M Tris, pH 8.5, 50 mM MgCl_2), and 16 μl of the 10X diluted labeled oligonucleotide. After incubation at 56°C for 1 hour, the annealing mixture was further mixed with 10 μl of 0.1 M DTT, 9 35 μl of 0.1 M Tris, pH 8.0, 2 μl of a solution containing 25 mM of each of the four deoxyribonucleotides, 2 μl of Klenow fragment (6 U/ μl , BRL), and 136 μl of distilled water. This

mixture was incubated at 37°C for 45 minutes and then at 65°C for 10 minutes. NaCl was then added to a final concentration of 50 mM and 50 units of restriction enzyme PvuII were added. After 4 hours of incubation at 37°C, 5 μ l of 0.5 mM EDTA was added and the mixture was extracted with phenol/chloroform and precipitated with ethanol. The pellet was resuspended in 25 μ l of sequencing dye solution, heated at 90°C for 10 minutes, and loaded onto a 5% polyacrylamide sequencing gel. After electrophoresis, a DNA band was detected by autoradiography and eluted from the gel by the "crush and soak" method.

S1 nuclease mapping. The single-stranded probe prepared above was resuspended in distilled water, and an aliquot was re-precipitated with 28 μ g of each test cellular RNA. The pellet was resuspended in 30 μ l of hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide), and incubated at 30°C for 15 hour. A 300 μ l solution containing 30 mM sodium acetate, 0.1 M NaCl, 2 mM ZnCl₂, 6 μ g denatured calf thymus DNA, and 150 units of mung bean nuclease (New England Biolabs) was then added. After 1 hour of incubation at 37°C, the mixture was precipitated, resuspended in sequencing dye solution, heated at 90°C for 10 minutes, and loaded onto an 8% sequencing gel. The probe was also chemically sequenced for the purine nucleotides and electrophoresed alongside the mung bean nuclease digested products.

Anchored PCR. The procedure used for cloning the 5' ends of plastin cDNAs with the anchored PCR method is described in Lin et al., *Mol. Cell Biol.* 10:1818 (1990). That procedure was used with the following modifications. (i) For reverse transcription, the procedure as described above in primer extension was used. (ii) For PCR, a downstream primer closer to the 5' ends of the L-plastin mRNA (247 bp from the 5' end of the existing cDNA clone as described in ref. Lin et al., *Mol. Cell Biol.* 10:1818 (1990) was used. (iii) The PCR products were cloned directly (after end-filling in and kinase reactions) into M13mp8 vector without restriction enzyme digestion or linker ligation.

Construction of the expression vector pHLPPr-1-neo and β -galactosidase reporter genes. An L-plastin gene promoter-based expression vector, pHLPPr-1-neo, was constructed by replacing the 4.3 kb EcoRI-HindIII fragment containing the 5 β -actin gene promoter in p β APr-1-neo Gunning et al., *Proc. Natl. Acad. Sci.* **84**:4831 (1987) with a 5.1 kb EcoRI-HindIII fragment (see Table 1) which was isolated from L-plastin genomic clone pg305.

TABLE 3

Comparison of the activities of the L-plastin and β -actin promoters in transfectant cell lines using B-galactosidase as a reporter enzyme

5	CELL TYPE	PROMOTER	TRANSFECTION TRIAL #	G418 RESIST. COLONIES	%+
<u>βgal+/total</u>					
Human Tumor derived cell lines:					
10	HuT-14 (fibrosarcoma)	L-plastin	1	19/53	35.8
			2	9/35	25.7
			3	25/96	26.0
			4	23/79	29.1
			total	76/263	28.9
15		β -actin	1	62/113	54.9
			control (pH β Apr-1-neo)	1	0/112
20	HT1080 (fibrosarcoma)	L-plastin	1	15/50	30.0
			2	12/43	27.9
			3	28/65	43.1
			total	55/158	34.8
25		β -action	1	58/82	70.1
30	HOS (osteosarcoma)	L-plastin	1	2/38	5.3
			2	2/52	3.8
			3	3/37	8.1
			total	7/127	5.5
35		β -actin	1	35/100	35.0
40	MG63 (osteosarcoma)	L-plastin	1	0/17	0
			2	1/27	3.7
			3	0/23	0
			4	0/36	0
			total	1/103	1.0
45		β -actin	1	18/71	25.4

5	RD (rhabdomyo- sarcoma)	L-plastin	1	0/185	0
			2	0/176	0
			3	0/192	0
			total	0/553	<0.2
10		β -actin	1	65/210	31.0
			2	55/177	31.1
			3	69/226	30.5
			total	189/613	30.8

Normal human fibroblast:

15	BC	L-plastin	1	0/62	0
			2	0/52	0
			3	0/51	0
			total	0/165	<0.6
		β -actin	1	1/2	50.0
			2	3/5	60.0
			3	6/20	30.0
			4	5/16	31.3
			5	7/24	29.2
			6	7/43	16.3
			total	29/110	26.4

SV40-transformed cell lines:

20	Wi-38VA13 (embryonic lung fibroblast)	L-plastin	1	3/240	1.3
			2	7/2136	0.3
			3	0/2241	0.4
			4	14/2069	0.7
			total	34/6686	0.5
25		β -actin	1	285/759	37.5
30		None	1	0/251	0
35	Wi-26VA4 (embryonic lung fibroblast)	L-plastin	1	3/2893	0.01
			2	7/3172	0.02
			3	9/3043	0.03
			total	19/9108	0.02
				β -actin	1

This L-plastin genomic fragment contained 4.2 kb of 5'-flanking sequence, the first exon, and 0.8 kb of the first intron. By inserting the *E. coli* β -galactosidase (*lacZ*) gene in the HindIII site, downstream from the L-plastin promoter, a reporter plasmid, pHLPPr- β gal-neo, was generated and used in the following experiments to evaluate the cell-type specificity of the L-plastin promoter.

For comparison with the L-plastin promoter, the HindIII DNA fragment encoding *E. coli* β -galactosidase was also inserted into the HindIII site in the polylinker of the β -actin promoter expression vector, p β APr-1-neo, to generate the plasmid p β APr- β gal-neo.

Selection for stable transformants. Sub-confluent diploid fibroblasts (BC strain; 1×10^6 to 1.5×10^6 cells per 100-mm diameter culture dish) in 9 ml of culture medium were transfected with 10 μ g of plasmid DNA by the calcium phosphate precipitation technique Leavitt et al., *Mol. Cell. Biol.* 7:2457 (1987). The culture medium was replaced after six to fifteen hours with fresh medium and then the cells were incubated for three days. All other cell lines were transfected as above except that transfections were done in 60 mm diameter culture dishes (using the same number of cells) with 5 μ g of DNA per dish and after replacement of the medium, cells were incubated for 24 to 36 hours.

To select G418-resistant colonies of normal fibroblasts, transfected diploid cells were then incubated in medium containing 600 μ g G418 (Gibco-BRL) per ml for six to seven days after which the drug was omitted for up to 12 days after transfection initiation at which time colonies were assayed for β -galactosidase activity.

To select G418-resistant colonies of the immortal cell lines, transfected cells were trypsinized and divided into two or three 100 mm dishes per each transfection. After 18 to 24 hours, the culture medium was adjusted to 800 μ g G418 per ml and cultures were incubated for eight to twelve days to allow colony development. The G418-supplemented medium was replaced every three to four days.

β -Galactosidase assay in situ. Expression of recombinant *E. coli* β -galactosidase was used as a reporter in a G418-resistant colony assay to determine the activity of the L-plastin promoters and to compare its activity with that of the β -actin promoter. This colony assay is a measure of the ability of the promoter to direct stable constitutive expression of a protein over multiple cycles of cell division. Histochemical staining for β -galactosidase expression in transfected cultured cells was performed according to the procedure of Sanes et al. *EMBO J.* 5:3133 (1986) and MacGregor et al. *Somatic Cell Mol. Genet.* 13:253 (1987) who characterized colonial expression of β -galactosidase using recombinant vectors.

Cells were first rinsed free of culture medium with phosphate-buffered saline (PBS), then fixed in chilled PBS supplemented with 0.2% glutaraldehyde for 5 minutes. Fixed cells were then rinsed three times with PBS. To develop the β -galactosidase histochemical stain, cells were incubated to 18 hours in the CO₂ incubator at 37°C with 5 to 10 mls of PBS (pH 7.2) supplemented with 5 mM potassium ferricyanate, 5 mM potassium ferrocyanate, 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal; BRL) from a 40X stock solution in DMSO, 2 mM MgCl₂, and 0.02% NP-40. Plates containing fixed and stained colonies were stored at 4°C in 90% glycerol/10% PBS, and examined for blue stained cells using an inverted light microscope with a 10X objective.

The uniformity of β -galactosidase activity in each cell in the colony as well as the percentage of cells in the colony that exhibited β -galactosidase expression were also useful in assessment of the stability of constitutive expression. In all cell lines tested using this colony assay, resulting colonies exhibited a wide range in levels of β -galactosidase expression as indicated by the differing amounts of blue color developed by the β -galactosidase-catalyzed reaction.

Although most colonies that exhibited β -galactosidase activity uniformly expressed this activity in most of the cells of a positive colony, some β -galactosidase-positive

colonies exhibited mosaic expression of β -galactosidase positive and β -galactosidase negative cells within the same colony. Therefore, β -galactosidase-positive colonies were defined as those that exhibited β -galactosidase activity in at least 20% of the colonial cells.

To confirm the expression of the β -galactosidase protein, individual HuT-12 and rat-2 colonies transfected with the plasmid pH β Apr- β gal-neo were isolated and cultured in duplicate cultures. One of each set of duplicate cultures was tested for β -galactosidase expression to identify the clonal cultures that expressed the highest cellular frequency and quantitative level of β -galactosidase activity. The most active clones for each cell type were recloned, and the resultant subclones were then retested to identify almost pure populations of cells expressing β -galactosidase abundantly.

These clonal cells were then examined to verify the identity of the E. coli β -galactosidase product using 2-D gels and 2-D gel Western blots. The β -galactosidase product (120,000 M_r, pI 5.2) was identified in subclonal HuT cells by the following criteria: its co-migration in the 2-D gel with purified unlabeled E. coli β -galactosidase and by its binding of anti-E. coli β -galactosidase antibody in a two dimensional gel Western blot. Identical results were obtained with rat-2 colonial cells expressing β -galactosidase.

The growth-selectable neomycin-resistance gene in pSV2-neo and pH β Apr-1-neo was used to select mammalian cell colonies that co-expressed a recombinant gene driven by the β -actin promoter. The protein product of the native human mutant β -actin gene was co-expressed in 4 out of 9 (44%) of the colonies produced by G418-resistant diploid human fibroblasts and 12 out of 17 (71%) of the colonies produced by G418-resistant HuT-12 fibrosarcoma cell line.

In another study both tubulin and actin antisense RNA transcripts were detected in 3 out of 5 (60%) and 4 out of 8 (50%) of the colonies produced by G418-resistant HuT-12. In a third study, expression of a recombinant human tissue plasminogen activator (tpa) gene was observed in 4 out of 8

(50%) of the colonies produced by G418-resistant diploid human fibroblasts and 12 out of 17 (71%) of the colonies produced by G418-resistant HuT-12. The results of these three independent studies indicate the expected frequency for co-expression of a 5 recombinant gene driven by the β -actin promoter (in colonies selected for G418-resistance) at approximately 47% for diploid human fibroblasts and about 66% for HuT fibroblasts.

Labeling and two dimensional polyacrylamide gel electrophoresis of cellular proteins. All cell types in high-10 density sub-confluent monolayers were labeled for 4-hrs with [³⁵S]methionine and samples prepared for electrophoresis as previously described Leavitt et al., *Mol. Cell Biol.* 7:2457 (1987). Two dimensional gels were also produced and analyzed as previously described Leavitt et al., *Mol. Cell Biol.* 7:2457 15 (1987). Western blotting of polypeptides separated in two dimensional gels has been described previously Varma et al., *Exp. Cell Res.* 173:163 (1987). Rabbit anti- β -galactosidase used at 1:500 dilution was purchased from Clontech (Palo Alto, CA).

20

EXAMPLE 2

Identification of the L-plastin Promoter

A 6 kb HindIII fragment in genomic clone pg305 was found to contain the 5' end of L-plastin cDNA. The sequence of a 25 3.2 kb region from a ScaI site to the downstream HindIII site is shown in Table 2 with nucleotide +1 representing the first transcription start site. Initial inspection of the sequence revealed that immediately adjacent to the 5' end of the cDNA (exon 1) there was a TATAAA sequence at -10 bp, an Sp-1 30 binding sequence at 43 bp, a sequence of 18 A nucleotides at -112 bp, and an AP-1 binding site at -536 bp, but no apparent CCAAT sequence.

By searching the sequence with a computer database of transcription factor binding sequences, four regions were 35 identified upstream from the TATAAA box at -104 bp, at -802 bp, at -1570 bp, and at -2082 bp (Table 2) that represented the consensus binding motif for transcription

factor Ets-1 (consensus sequence is $^G/C^A/C\text{GGA}^A/T\text{G}^T/C$; Faisst and Meyer, *Nucleic Acids Res.* 20:3 (1992), which is specifically expressed in hemopoietic cells.

By screening the nucleotide sequence data bank (GenBank), 5 the Ets-1 core motif AGGAAG nearest the L-plastin promoter (-104 bp, or -88 bp upstream from the TATAAA box) was found to be homologous to Ets-1 motifs next to the promoters of other genes that encoded hemopoietic-specific proteins such as Ets-1, interleukins 2, 3, 4, and 6, G-CSF, GM-CSF, the T-cell 10 receptor α and β chains. In addition, this core motif was found in the LTR sequences of the lymphotropic viruses HIV-1 and HTLV-1.

Two regions related to the progesterone responsive element at -1127 bp (AGAACAGTTTGGTTT; Table 2, indicated as 15 PR) and at -1660 bp (AGAACACTGTGCTTT; Table 2, indicated as PR) which are half-palindromes of the consensus progesterone responsive element (AGAACAN₃TGTTCT) and one region related to the estrogen responsive element at -73 bp (ATTTCACTGTGACCT; Table 2, indicated as ER) which is a half-palindrome of the 20 consensus estrogen responsive element (AGGTCAN₃TGACCT) were also found. The estrogen binding motif was flanked by the Ets-1 motif 16 bp upstream and the Sp1 motif 24 bp downstream.

Both the estrogen responsive element and the progesterone responsive element are functional since these hormones induce 25 L-plastin expression in cell cultures of hormonally responsive reproductive tissues. Using PCR with primers homologous to these estrogen responsive element and progesterone responsive element sequences established that these specific elements are unique to the L-plastin gene because only a single 30 amplification product homologous to the L-plastin sequence between the estrogen responsive element and the upstream progesterone responsive element could be amplified.

Since estrogen up regulates progesterone receptor synthesis and progesterone down regulates the progesterone 35 receptor synthesis, the presence of both steroid receptor enhancers indicates that either hormone receptors or both can act in concert with Ets-1 to regulate the L-plastin gene

either positively or negatively. The GenBank database was searched for other genes that contained both Ets-1 and steroid responsive elements and no other genes with this combination of transcriptional control elements was identified.

5

EXAMPLE 3

Evaluation of Promoter Activity

Using transient transfection CAT assays with HuT-14 cells, the promoter activity in five DNA fragments spanning 10 the TATAAA box was examined (Table 4).

TABLE 4

<u>Fragment No.</u>	<u>Restriction Sites</u>	<u>CAT Activity</u>
1	PvuII/ScaI (PS)	9.7
15 2	HincII/ScaI (CS)	3.6
3	ScaI/ScaI (SS)	3.4
4	PvuI/HindIII (PH)	9.7
5	EcoRI/HindIII (EH)	1.2
6	β -actin	9.835

20

The two fragments (PS and PH) having the shortest sequence (180 bp) upstream from the TATAAA box showed the strongest promoter activity, which was nearly equal to the activity of the β -actin promoter. These two fragments 25 differed only by the presence of additional 29 bp of the first exon and 717 bp of the first intron in the longer fragment PH. These additional sequences apparently had no effect on the promoter activity.

Fragments SS and CS differed from fragment PS by having 30 longer upstream sequences, which appeared to account for a 63-65% reduction in promoter activity. The longest fragment (EH) differed from fragment PH only by having a 4 kb additional upstream sequence, which resulted in a reduction of promoter activity by approximately 88%. This lowest level of

L-plastin promoter activity was consistent with the difference in the levels of protein synthesis between L-plastin and β -actin in HuT-14 cells.

These results indicate that the L-plastin gene contains a very potent transcriptional promoter which is attenuated by upstream negative regulatory elements.

The high activity of the smallest promoter fragment indicates that in some instances during transfection the recombinant promoter became truncated leading to removal of upstream elements that attenuate the activity of the promoter in these L-plastin-negative cell lines.

The finding of steroid hormone response elements near the L-plastin promoter indicates that expression of L-plastin in hemopoietic cells may, under some circumstances, be subject to hormonal control either locally (in reproductive tissues that synthesize and secrete steroid hormones) or systemically by the circulating levels of these hormones. In this regard, progesterone receptors have been detected in a small sub-population of peripheral blood lymphocytes of non-pregnant women and the abundance of this hemopoietic sub-population has been shown to increase as much as 30-fold in the late stages of pregnancy when progesterone levels become greatly elevated. That reproductive hormone levels influence expression of a fundamental hemopoietic architectural protein such as L-plastin demonstrates the role of hormones in control of immune system by their regulation of hemopoietic-specific gene expression during pregnancy. Therefore, the L-plastin promoter region can be used to engineer steroid hormone-regulated expression of beneficial proteins and RNA in steroid hormone-responsive tissues and in steroid hormone-responsive hemopoietic cells during pregnancy.

The finding of four Ets-1 binding motifs upstream from the TATAAA box is consistent with the hemopoietic specificity of L-plastin gene expression in normal cells because the Ets-1 binding motif is found near the promoters of many other genes that are expressed in a hemopoietic cell-specific manner. The presence of the four Ets-1 elements explains the high stable

rate of constitutive expression observed in hemopoietic cells and the stringent repression of the L-plastin promoter observed in non-hemopoietic cells.

5

EXAMPLE 4

Determination of Transcription Start Sites

The transcription start site(s) were determined by primer extension and S1 (mung bean nuclease) mapping methods. In the primer extension experiment, a 25-nucleotide primer
10 corresponding to the 3' end of exon 1 (Table 2) was used. The primer could only be extended with RNA templates prepared from the two cell lines that expressed L-plastin, i.e. HuT-14 fibrosarcoma cells and CEM lymphoblastoid cells, but the primer could not be extended with RNA from diploid KD
15 fibroblasts which do not express L-plastin.

The extension products were of multiple lengths, indicating that the L-plastin gene had multiple transcription start sites. One start site appeared to be 10 nucleotides downstream from the TATAAA box (nucleotide +1).

20 Using S1 mapping, again the genomic probe was specifically protected by RNAs prepared from the two cell lines that expressed L-plastin, i.e. HuT-14 fibrosarcoma cells and CEM lymphoblastoid cells, but the genomic probe could not be protected with RNA from diploid KD fibroblasts which do not
25 express L-plastin.

Consistent with the findings using primer extension, the protection products were also of multiple lengths. However, there were some differences in the banding patterns between the primer extension and the nuclease mapping products. These
30 differences may result from local DNA secondary structure which may influence the movement of reverse transcriptase along the template.

More importantly, the two techniques gave very similar results that were in agreement in locating transcription start
35 sites very close to the TATAAA box. The results of both primer extension and S1 mapping also indicated that the most

preferred sites were located between 91 bp and 111 bp downstream from the TATAAA box (+81 to +101).

Although transcription usually starts approximately 30 bp downstream from the TATAAA box, the above mapping results 5 indicated that transcription of the L-plastin gene could start much closer to the TATAAA box. To confirm this, anchored PCR was performed as described in Example 1 for cloning of the 5' ends of the plastin cDNAs. Several PCR products were isolated by cloning and sequenced. The longest clone contained as its 10 5' sequence the nucleotide sequence starting at 10 bases downstream from the TATAAA box. For convention, this nucleotide is referred to as position +1 in the transcription product of the L-plastin gene (Table 2).

The results of primer extension, S1 mapping, and anchored 15 PCR/cDNA cloning demonstrates that (i) transcription initiation occurs at multiple sites between 10 bp and 111 bp downstream from the TATAAA box, (ii) the most frequently used transcription initiation sites are located 91 bp to 111 downstream from the TATAAA box, and (iii) patterns of 20 transcription initiation are virtually identical in CEM lymphoblastoid cells and in HuT fibrosarcoma cells.

EXAMPLE 5

Recombinant L-plastin Promoter Activity in Normal and Neoplastic Fibroblasts

25 *E. coli* β -galactosidase expression from a recombinant *E. coli* lacZ gene as a reporter was used to measure the ability of various cell lines and strains to support constitutive activation of the L-plastin promoter. This assay 30 permits assessment of the percentage of cells in individual G418-resistant colonies that co-express β -galactosidase and assessment of uniformity of expression under the control of a mammalian gene promoter. An L-plastin promoter expression vector, pHLPPr-1-neo, was constructed using the largest 35 promoter fragment characterized in Table 4 because this fragment contained potential cis-acting regulatory elements that were greater than 1 kb upstream from the TATAAA box. The

β -galactosidase reporter gene (lacZ) was inserted into this plasmid at the 3' end HindIII site of the promoter (Table 4) to create the plasmid pHLPPr- β gal-neo. The β -actin promoter was chosen for comparison with the L-plastin promoter because
5 this promoter is a strong constitutive promoter which is active in all replicating cell types; thus, the β -galactosidase reporter gene was also inserted into the plasmid pH β APr-1-neo at the HindIII site of its polylinker to create the plasmid pH β APr- β gal-neo (as described in
10 Example 1).

When the β -actin promoter was used to drive expression of β -galactosidase, the co-expression frequencies for β -galactosidase in G418-resistant colonies approached the high frequencies achieved for the native mutant β -actin gene,
15 antisense transcripts, and the recombinant tpa gene as described in Example 1.

Colonies that exhibited β -galactosidase expression under the control of the L-plastin and β -actin promoters were identified. Table 5 compares the co-expression frequencies of
20 these two promoters in driving β -galactosidase expression in various human tumor-derived cell lines, a diploid human fibroblast strain, and two SV40-transformed cell lines. In Table 5, relative activity is the activity of L-plastin divided by the activity of β -actin.

25

TABLE 5

5 **Relative activities of the L-plastin and β -actin promoters
in transfectant cell lines using β -galactosidase
as a reporter enzyme**

human cell strain	RELATIVE PROMOTER ACTIVITY (% positive clones)			
	L-plastin expression	L-plastin promoter	β -actin promoter	relative activity
10 HuT-14 fibrosarcoma	++	28.9	54.9	.53
HT1080 fibrosarcoma	++	34.8	70.1	.50
15 HOS osteosarcoma	+	5.5	35.0	.16
MG63 osteosarcoma	-	1.0	25.4	.04
Wi-38VA13	-	0.5	37.5	.013
Wi-26VA4	-	0.02	30.3	<.001
RD rhabdomyosarcoma	-	<0.02	30.8	<.001
20 diploid fibroblasts (BC)	-	<0.6	26.4	<.023

25 When β -galactosidase expression was driven by the β -actin promoter (in pH β APr- β gal-neo), 55% of the developing G418-resistant colonies exhibited synthesis of the dark blue β -galactosidase enzymatic product (as shown in Table 5). Detection of colonial cells that expressed the E. coli

30 β -galactosidase-catalyzed reaction was dependent upon transfection with a vector that contained the E. coli β -galactosidase gene since no G418-resistant cells or colonies were capable of producing a blue metabolite following transfection with pH β APr-1-neo which lacked the

35 β -galactosidase gene (Table 5).

Many of the β -galactosidase-positive colonies produced by pH β APr- β gal-neo were visible without a microscope, but many

more positive colonies which uniformly expressed the β -galactosidase-catalyzed reaction at a lower constitutive level were easily detectable microscopically. The co-expression frequency of β -galactosidase under the control of the L-plastin promoter in G418-resistant HuT-14 cells produced by transfection of pHLPPr- β gal-neo averaged 29% in four independent transfection trials (Table 5).

Using a second human fibrosarcoma cell line, HT1080, the co-expression frequency of β -galactosidase under the control of the L-plastin promoter in G418-resistant colonies averaged 35% in three separate transfection trials compared to 70% when the β -actin promoter drove β -galactosidase expression (Table 5).

Routinely, the color reaction catalyzed by β -galactosidase developed faster and produced a darker blue color when the β -actin promoter was used indicating that the β -actin promoter was the stronger of the two promoters as suggested by their relative activities in supporting CAT expression (Table 4).

The extent of the color reaction produced by the two promoters can be seen by comparison of the darker β -galactosidase-positive colonies and cells produced with the β -actin promoter with those colonies and cells produced with the L-plastin promoter. Thus, the lower colonial frequency of β -galactosidase co-expression with the L-plastin promoter is due to the lower level of constitutive activity (and thus lower sensitivity of the assay) compared to the β -actin promoter.

Twenty-six percent of the diploid human fibroblast (BC) colonies were positive for β -galactosidase activity when its expression was under the control of the β -actin promoter (Table 5). By contrast, diploid BC fibroblasts which do not express L-plastin failed to exhibit any detectable β -galactosidase expression with the L-plastin promoter in 165 G418-resistant colonies which developed in three transfection trials (Table 5).

Three additional tumor-derived cell lines - HOS (osteosarcoma), MG63 (osteosarcoma), and RD (rhabdomyosarcoma) - were tested for their ability to support the activity of the L-plastin promoter. HOS, which expresses a relatively low level of L-plastin protein and mRNA constitutively, exhibited a 5.5% β -galactosidase expression frequency when expression was under the control of the L-plastin promoter and a 35% expression frequency when expression was under the control of the β -actin promoter (Table 5). MG63 and RD, which do not express detectable L-plastin protein and mRNA, exhibited only one β -galactosidase-positive colony (1%) and no positive colonies ($< 0.2\%$), respectively, when expression was under the control of the L-plastin promoter in contrast to a 25% and 31% expression frequency, respectively, when expression was under the control of the β -actin promoter (Table 5).

Two SV40-transformed human fibroblast cell lines were also tested for their ability to utilize the L-plastin promoter constitutively. These cell lines, which do not express endogenous L-plastin gene (Wi-38VA13, Wi-26VA4), exhibited a low percentage of colonies that expressed β -galactosidase at low constitutive levels under the control of the L-plastin promoter, ranging from 0.01 to 0.5. In contrast, β -galactosidase expression under the control of the β -actin promoter ranged from 24% to 38% in these three cell lines (Table 5). The rare Wi-38VA13 and Wi-26VA4 colonies that expressed β -galactosidase with the L-plastin promoter were weakly stained and could only be detected by microscopic examination indicating low levels of β -galactosidase expression.

In Table 5 the relative frequency of G418-resistant colonies that expressed β -galactosidase under the control of the L-plastin promoter was compared among the seven immortal cell lines and normal diploid BC fibroblasts after normalization to the frequency of G418-resistant colonies that expressed β -galactosidase under the control of the β -actin promoter. This calculation takes into account the finding that differing cell strains express the co-selected gene at

different frequencies and allows the comparative rating of the differing cell types for their ability to support activation of the recombinant L-plastin promoter.

The two fibrosarcoma cell lines which expressed the 5 highest levels of L-plastin (HuT-14 and HT1080; Lin et al., *Mol. Cell Biol.* 8:4659 (1988) also exhibited the highest relative activity of the recombinant L-plastin promoter which was 0.50 to 0.53 as active as the recombinant β -actin promoter. HOS cells which expressed a lower, but detectable, 10 level of L-plastin exhibited reduced activity of the recombinant L-plastin promoter which was 0.16 as active as the β -actin promoter.

The four other immortal cell lines (MG63, RD, Wi-38VA13, and Wi-26VA4) which did not express detectable levels of 15 L-plastin polypeptide or mRNA exhibited very low relative activity of the recombinant L-plastin promoter ranging from 0.04 down to less than 0.001 as active as the β -actin promoter. The 0.04 rating for MG63 was based on the observation of only one positive colony out of 103 20 G418-resistant colonies examined while the other three cell lines gave much lower ratings based upon examination of much larger numbers of colonies. Finally, diploid BC fibroblasts which exhibited no expression of β -galactosidase under the control of the L-plastin promoter was rated by this method at 25 less than 0.023.

This analysis demonstrates that the frequency of constitutive activation of the recombinant L-plastin promoter is at least 12.5 times higher in cell lines that express the endogenous L-plastin gene at the highest levels compared to 30 cell lines that do not express the exogenous L-plastin gene. Furthermore, when these highly active fibrosarcoma cell lines were compared with L-plastin-negative cell lines that exhibited high cloning frequencies in G418, they exhibited between 40 and greater than 500 times higher frequency of 35 constitutive activation. The active fibrosarcoma cell lines were also at least 22 times more active than normal diploid BC fibroblasts.

These results indicate that activation of the L-plastin gene in tumorigenesis is governed by cis-acting elements present in the 5.1 kb EcoRI-HindIII L-plastin gene promoter fragment.

5

EXAMPLE 6

*Use of the L-plastin Promoter
to Determine Neoplastic Transformation*

An expression vector, designated pHLPPr-1-neo, was
10 constructed using a 5.1 kb genomic fragment containing the
L-plastin promoter and flanking sequences, as described in
detail in the Examples. Using β -galactosidase as a reporter
of activation of the L-plastin promoter, the activity of
L-plastin promoter was evaluated in a panel of fibroblastoid
15 cell types which included a normal fibroblast strain that did
not express detectable L-plastin, tumor-derived fibrosarcoma
strains which did or did not express L-plastin, and SV40-
transformed human fibroblasts which did not express L-plastin.

The frequency of constitutive activation of the
20 recombinant L-plastin promoter in transfected G418-resistant
cell colonies was significantly higher in those cell types
that expressed the endogenous L-plastin gene. (G418 is a
synthetic neomycin analogue.)

A second expression vector designated pHLPPr-neo was
25 constructed using the same 5.1 kb genomic fragment containing
the L-plastin promoter and flanking sequences as described in
detail in the Examples. Using the neomycin resistance gene
(neo) as the reporter of activation of the L-plastin promoter,
the activity of the L-plastin promoter was evaluated in a
30 panel of fibroblastoid cell types.

The frequency of development of colonial substrains which
were resistant to G418 following transfection of the
recombinant neo-resistance gene which was under the control of
the recombinant L-plastin promoter was significantly higher in
35 those cell types that expressed the endogenous the L-plastin
gene. Moreover, a significant number of the G418-resistant
sub-colonial cell strains that were derived from the parental

cell lines which did not express detectable levels of L-plastin exhibited detectable levels of expression of the endogenous L-plastin gene. Thus, by putting a growth selection marker under the control of the L-plastin promoter 5 and its natural regulatory elements, selection of cells which have activated the endogenous L-plastin gene either transiently or constitutively is facilitated.

This example demonstrates the utility of the recombinant promoter in investigation of mechanism(s) of neoplastic 10 transformation leading to activation of the L-plastin promoter region, and the potential role of hormonal regulation in activation of the L-plastin gene in tumorigenesis.

EXAMPLE 7

15 *Evaluation of Activation of the L-plastin Gene*

Since L-plastin is a common marker of human cancer cells, the frequency and nature of this gene activation event were of great interest. Therefore, to determine whether activation of 20 the L-plastin gene was more wide-spread than previously thought, the sensitive Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was used to examine L-plastin SV40-transformed fibroblast cell lines, tumor derived cell lines, and in a variety of diploid cell types for low level 25 activation of the L-plastin gene which were L-plastin negative by less sensitive methods.

RT-PCR Analysis RT-PCR analysis was performed as follows for detection of latent L-plastin expression. Cellular RNA 30 was isolated from cultured cells by the guanidine hydrochloride phenol-chloroform extraction method (Chomczynski et al, *Anal. Biochem.* 162: 156-159 (1987)). For reverse transcription, 50 ng of random hexamers was annealed to 5 μ g sample RNA and extended by Moloney Murine Leukemia Virus RNase 35 H-Reverse Transcriptase (Gibco BRL; Gaithersberg, MD) in a reaction volume of 10 μ l. A portion (1 μ l) of the cDNA was used in PCR which included 25 ng of L-plastin oligomer

(TGAAAGAACAATCAACAAA) as the upstream primer and 25 ng of L-plastin oligomer (TTAATGGAACCTGGTTGG) as the downstream primer in a reaction volume of 50 μ l containing 1.25 units of taq DNA polymerase (Gibco BRL; Gaithersberg, MD) and buffer 5 supplemented with 2 mM of MgCl₂ and 0.1 mM deoxynucleotide triphosphates.

The PCR was run in an Ericomp thermocycler for 35 cycles with each cycle consisting of 94°C, 30 seconds; 45°C, 40 seconds; 72°C, 40 seconds. After an additional 10 minute 10 incubation at 72°C, 10 μ l of the PCR reaction solution was electrophoresed in a 1.5 % agarose gel. After staining and photography, the DNA was transferred to a Duralose-UV membrane (Stratagene; La Jolla, CA) and hybridized with a ³²P-labeled L-plastin probe.

15 To test for trace levels of L-plastin expression, RT-PCR was performed as described above on RNA isolated from eight diploid human cell types (four fibroblast strains, mammary epithelial cells, skin keratinocytes, umbilical vein endothelial cells, and aortic smooth muscle cells), four 20 SV40-transformed human fibroblast strains (Wi38-VA13, Wi26-VA4, GM3022, and M1SV; Lin et al, *J. Biol. Chem.* 268:2781-2792 (1993)), and 12 tumor derived cell lines in which L-plastin expression was undetectable using two dimensional gel protein profiling or Northern blotting (Lin et 25 al, *J. Biol. Chem.* 268:2781-2792 (1993)).

The RT-PCR amplification products were electrophoretically resolved in a 1% agarose gel, trans-blotted onto nylon membranes and hybridized with an L-plastin cDNA probe to confirm their identities as amplified sequences 30 from the L-plastin mRNA for analysis by Southern blots as described above. These Southern blots were designated Blot 1 (described below), and the results of the full survey of L-plastin expression are summarized in Table 6 (below) which includes the additional cell types that were found previously 35 to express L-plastin more abundantly (Lin et al, *J. Biol. Chem.* 268:2781-2792 (1993)). Blot 1 was a Southern blot of

RT-PCR amplification products from normal and neoplastic human cells. The samples in the blot were as follows:

	<u>Sample</u>	<u>Cell Line</u>
5	1	Hep-G2 hepatic carcinoma
	2	SK-Hep-1 hepatic carcinoma
	3	A427, lung carcinoma
	4	ENDCA endometrial carcinoma
	5	RD rhabdomyosarcoma
10	6	blank
	7	WERI retinoblastoma
	8	Y79 retinoblastoma
	9	blank
	10	Wi38 diploid embryonic lung fibroblasts
15	11	
	12	Wi38-VA13 SV40-transformed fibroblasts
	13	Wi26-VA4 SV40 transformed fibroblasts
20	14	HuVEC umbilical endothelial cells
	15	ASM, aortic smooth muscle cells
	16	MRC5 diploid embryonic lung fibroblasts
	17	KER, skin keratinocytes
25	18	MEC, mammary epithelial cells
	19	blank
	20	AN3CA, endometrial carcinoma
	21	A204 rhabdomyosarcoma
	22	T130 rhabdomyosarcoma
30	23	MG63, osteosarcoma
	24	C33A, cervix carcinoma
	25	M1SV, SV40-transformed fibroblasts
	26	GM3022, SV40-transformed fibroblasts
	27	T130 rhabdomyosarcoma

35

The origin of each strain of cells used in Blot 1 is given in Lin et al, *J. Biol. Chem.* 268:2781-2792 (1993).

TABLE 6					
Cell Types		L-plastin Expression			
<u>Normal cells</u>	<u>Strain</u>	None	Trace	Low	High
5 leukocytes (Lin et al, <i>J. Biol. Chem.</i> 268:2781-2792 (1993))					+
10 uterine stromal cells	primary				+
	late passage			+	
15 fibroblast cells	MRC5	+			
	Wi38	+			
	KD	+			
	BC	+			
15 aortic smooth muscle cells		+			
umbilical vein endothelial cells		+			
20 mammary epithelial cells		+			
	skin keratinocytes	+			

<u>Neoplastic Cell Lines</u>						
5	hemopoietic tumors (n=9, Lin et al, <i>J. Biol. Chem.</i> 268:2781-2792 (1993))					+
10	SV40 transformed fibroblast lines (n=5)	MRC5-SV2				+
		Wi38-VA13		+		
		Wi26-VA4		+		
		M1-SV		+		
		GM3022		+		
15	bladder carcinoma (n=1)	T24				+
	cervical carcinoma (n=2)	HT-3				+
		C-33A		+ ¹		
20	choriocarcinoma (n=2)	BeWo				+
		JAR				+
	colon carcinoma (n=2)	CaCo-2				+
25		HT-29			+	
	endometrial carcinoma (n=2)	ENDCA		+		
		AN3CA	+			

	epidermal carcinoma (n=1)	A431				+
	fibrosarcoma (n=4)	8387 (HuT series)				+
		HT1080				+
5		SW684				+
		HS913A				+
	kidney carcinoma (n=1)	A704			+	
10	leiomyosarcoma (n=1)	sarcoma-2				+
	liposarcoma (n=1)	SW872			+	
	liver carcinoma (n=2)	Hep-G2	+			
		SK-Hep-1		+		
15	lung carcinoma (n=1)	A427		+		
	mammary carcinoma (n=4)	BT-20				+
		HTB-130				+
20		HTB-132				+
		MCF-6			+	
	melanoma (n=1)	MeWo			+	
	osteosarcoma (n=2)	HOS			+	
		MG63		+ ²		
25	ovarian carcinoma (n=8)	OVCA-420				+
		OVCA-433			+	

	(primary ovarian carcinomas)	strain A				+
		strain B				+
		strain C				+
5		strain D				+
		strain E				+
		strain F				+
	pancreatic carcinoma (n=1)	capan-2			+	
10	retinoblastoma (n=2)	WERI-RB-1		+		
		Y79		+		
	rhabdomyosarcoma (n=3)	A204	+			
15		RD		+		
		T130-1	+			
	squamous cell carcinoma (n=1)	SSC83-01-82				+
	stomach carcinoma (n=1)	HS746T				+
20	Total non-leukocyte transformations		4	12	8	24
	% of transformations		8%	25%	17%	50%
25						

¹ positive in 1 of 2 trials

² positive in 2 of 3 trials

In the table, the characterization of L-plastin expressions are as follows: "none" means not detectable by 30 RT-PCR; "trace" means detectable by RT-PCR only; "low" means

detectable by two dimensional gel or Northern blot analysis (Lin et al, *J. Biol. Chem.* **268**:2781-2792 (1993)); "high" means abundant expression (Lin et al, *J. Biol. Chem.* **268**:2781-2792 (1993))

5 RNA isolated from the diploid cell types was uniformly negative for the L-plastin mRNA/RT-PCR product (Blot 1, samples 10 and 14-18), while RNA from all four SV40-transformed fibroblasts supported the significant amplification of two L-plastin mRNA/RT-PCR products, one at
10 the predicted size of 1160 bp and a second amplification product of approximately 500 bp in size (Blot 1, Table 6). The smaller amplification product may be generated by primer annealing to an alternative sequence in the duplicated actin binding domain of L-plastin (de Arruda et al, *J. Cell Biol.*
15 **111**:1069-1079 (1990)).

RNA from diploid MCR5 fibroblasts (Blot 1, sample 16), the parent strain of MRC5-SV2 which synthesizes abundant levels of L-plastin (Lin et al, *J. Biol. Chem.* **268**:2781-2792 (1993); Celis et al, *Electrophoresis* **11**:1072-1113 (1990)), and
20 RNA from diploid Wi38 fibroblasts (Blot 1, sample 10), the parent strain of Wi38-VA13 (Blot 1, sample 12), exhibited no L-plastin mRNA transcripts detectable by RT-PCR. In addition, 8 out of 12 of the human tumor-derived cell lines that were thought to be negative for L-plastin expression (Lin et al, *J.*
25 *Biol. Chem.* **268**:2781-2792 (1993)) exhibited an L-plastin amplification product which indicated that the L-plastin gene was weakly active in many of the "negative" neoplastic cell lines.

In all, 92% of the human transformed cell strains
30 surveyed exhibited widely varying degrees of L-plastin expression of which 50% exhibited very abundant L-plastin expression which approached the level of L-plastin synthesis in hemopoietic cells (Table 6; Lin et al, *J. Biol. Chem.* **268**:2781-2792 (1993); Goldstein et al, *Cancer Res.* **45**:5643-
35 5647 (1985)). Also, all of the leukemia cell lines exhibited abundant L-plastin expression in sharp contrast to rodent

leukemia cell lines which uniformly lacked L-plastin synthesis (Lin et al, *J. Biol. Chem.* 268:2781-2792 (1993)).

This study demonstrated that the L-plastin gene is active in most human transformed cell strains, including strains 5 which were previously characterized as L-plastin negative.

EXAMPLE 8

Expression of a Neo Gene under the Control of the L-Plastin Promoter

The ability of a neo gene constructed with the L-plastin promoter, HuLPPr-neo, to express neo and therefore select L-plastin-positive clonal subpopulations was studied as described below.

15

Construction of the expression vector, pHuLPPr-neo All plasmids were grown in *E. coli* host XL-blue, and recombinant plasmid DNA was extracted by a standard alkaline lysate procedure. The plasmid, pH β APr-1, which contains human β -actin gene promoter has been described in Gunning et al, *Proc. Natl. Acad. Sci.* 84: 4831-4835 (1987). The plasmid, pNEO, which contains 1.5 kb neo-gene fragment was purchased from Pharmacia (Alameda, CA). DNA restriction fragments separated on agarose gels were purified using Gene Clean kit (Bio 101). All restriction enzymes were from New England Biolabs (Beverly, MA), and T4 ligase was from Gibco BRL (Gaithersburg, MD).

To construct the expression vector which utilized the L-plastin promoter, the 4.3 kb EcoRI-HindIII fragment of the β -actin gene promoter was excised from pH β APr-1 and replaced with the 5.1 kb, EcoRI-HindIII fragment containing the L-plastin gene promoter, 4.2 kb of the 5'-flanking sequence, the first exon, and 0.8 kb of the first intron (Lin et al, *J. Biol. Chem.* 268:2793-2801 (1993)). The 1.5 kb neo cDNA HindIII-BamHI fragment was excised from pNEO, and inserted at the HindIII and BamHI sites in the plasmid polylinker adjacent

to the 3' end L-plastin promoter fragment to produce pHuLPPr-neo.

Cell Culture and Transfection The origin of all cell lines used in this study have been described in Lin et al, *J. Biol. Chem.* **268**:2781-2792 (1993). Cells were maintained in minimal essential α -medium (MEM- α) supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml). Transfections of plasmid DNA were accomplished using 9 μ g DNA for 2-8 x 10⁵ cells in 60 mm diameter culture dish using the calcium phosphate precipitation method (Graham et al, *Virology* **52**:456-467 (1973); Kingston et al, *Mol. Cell. Biol.* **4**:1970-1977 (1984)).

For each cell line, four individual transfections were conducted. The culture medium was replaced after 16-18 hours with fresh medium, and cells were incubated further for 48-72 hours until they become confluent. Cells in a 60 mm culture dish were trypsinized and seeded into three 100 mm diameter culture dishes per transfected culture overnight. Geneticin (G418) was then added to the medium at a concentration of 400 μ g G418 per ml for the selection of stably transfected subclonal cell lines. The number of colonies which formed in a 100 mm culture dish were counted following fixation with methanol and staining with 0.1% Giemsa (Eastman Kodak; Rochester, NY).

The plasmid, pH β APr-1-neo, which contains an SV40-neo gene (Gunning et al, *Proc. Natl. Acad. Sci.* **84**: 4831-4835 (1987)) was used for control transfections. In order to correct the differences in the transfection efficiencies between differing cell lines, the frequency of colony formation in pHuLPPr-neo transfected cells was divided by the frequency achieved with pH β APr-1-neo conducted in parallel. G418-resistant colonies were isolated by trypsinization, and transferred to replicate 15 mm wells of 24 well culture dishes for ³⁵S-methionine labeling and further culturing. Subconfluent cell monolayers were labeled in 24 well tissue culture dishes with ³⁵S-methionine for 6-7 hours. Samples were prepared and two dimensional gels were analyzed as described

in Leavitt et al, *Molec. Cell. Biol.* 6: 2721-2726 (1986) and Leavitt et al, *Molec. Cell. Biol.* 7: 2467-2476 (1987).

Detection of L-plastin protein in individual subclones

5 selected with the L-plastin promoter Since the recombinant L-plastin promoter exhibited a relatively high promoter activity in cell lines that had high endogenous L-plastin promoter activity (Lin et al, *J. Biol. Chem.* 268:2793-2801 (1993)), this specificity of the recombinant promoter was used
10 to select subpopulations of cells from the L-plastin "negative" cell lines by using the L-plastin promoter to drive the expression of neo and resistance to the antibiotic G418.

The pHuLPPr-neo plasmid DNA was then used to transfect HuT-14 fibrosarcoma cells, which expressed L-plastin
15 abundantly, and three cell lines (MG63 osteosarcoma, RD rhabdomyosarcoma, and Wi38-VA13) that exhibited only trace levels of L-plastin mRNA detectable by RT-PCR (Blot 1, Table 6).

The osteosarcoma-derived MG63 cell line produced no
20 G418-resistant colonies from 5.2×10^5 transfected cells in three independent transfection trials with the HuLPPr-neo gene while HuT-14 fibrosarcoma cell line (the L-plastin positive variant cell line of the 8387 fibrosarcoma) produced on average 568 colonies from 3.4×10^5 transfected cells. The rhabdomyosarcoma-
25 derived RD cell line produced an average of 41 G418-resistant colonies from 4.6×10^5 transfected cells, and the SV40-transformed fibroblast cell line, Wi38-VA13, produced an average of 101 G418-resistant colonies from 7.4×10^5 transfected cells. Two other human rhabdomyosarcoma cell lines, T130 and
30 A-204 (Lin et al, *J. Biol. Chem.* 268:2781-2792 (1993)), and a second SV40-transformed human fibroblast cell line GM3022 (Lin et al, *J. Biol. Chem.* 268:2781-2792 (1993)) also failed to produce G418-resistant colonies following transfection with the HuLPPr-neo gene.

35 The cells of these G418-resistant colonies produced with HuLPPr-neo and the SV40-neo gene were examined for L-plastin expression by trypsinization of the primary colony and

duplicate plating of individual colonial cells which provided one culture for immediate labeling with ^{35}S -methionine and the second culture for further propagation of the subclonal cell line and characterization of its properties. The clonal cells were estimated to have achieved about 14 population doublings from the start of colony development through to protein labeling (expansion of 1 cell to 32,000 cells). Labeled proteins from the individual G418-resistant clones were resolved by high resolution two dimensional gel electrophoresis of trace ^{35}S -methionine labeled L-plastin polypeptide in *HuLPPr-neo* selected subclones. The findings in separate two dimensional gels with three subclonal strains from Wi38-VA13 and two RD subclonal strains are described below:

15

	<u>Gel</u>	<u>Cell Line</u>
	1	Wi38-VA13 subclone D-1.4
	2	Wi38-VA13 subclone B-1.1
	3	Wi38-VA13 subclone D-2.8
20	4	RD subclone RD-5
	5	RD subclone RD-30.

Polypeptides x and T, the two T-plastin polypeptides (Feinberg et al, *Anal. Biochem.* 137:266-267 (1983) were used in each gel as reference polypeptides for the location of L-plastin in the gel pattern.

Gel 1 was a protein profile which showed that one Wi38-VA13 subclone D-1.4 exhibited no apparent synthesis of L-plastin because it lacked a polypeptide species at the L-plastin electrophoretic position. By contrast, gels showing the proteins synthesized by the four other clonal strains B-1.1 (Gel 2), D-2.8 (Gel 3), RD-5 (Gel 4), and RD-30 (Gel 5) each exhibited a trace level of polypeptide in the precise electrophoretic position of L-plastin (see Lin et al, *J. Biol. Chem.* 268:2781-2792 (1993)) relative to the position of polypeptide x and the two T-plastin polypeptides (Gel 1 Lane A; Lin et al, *J. Biol. Chem.* 268:2781-2792 (1993)).

Although L-plastin mRNA and protein had not been previously detected in either of these cell lines by conventional Northern blotting and repeated two dimensional gel protein profiling (Lin et al, *J. Biol. Chem.* **268**:2781-2792 (1993)), 9 out of 12 of the Wi38-VA13 subclones tested and 18 out of 19 of the RD subclones clones tested exhibited a trace level of L-plastin synthesis by the appearance of a polypeptide at the discrete electrophoretic position of L-plastin. This barely detectable level of L-plastin synthesis was too low to be confirmed by Western blotting with anti-plastin antibody. However, expression of L-plastin from the endogenous genes of other cells (Lin et al, *J. Biol. Chem.* **268**:2781-2792 (1993), Goldstein et al, *Cancer Res.* **45**:5643-5647 (1985)) and from its cDNA transfected into Wi38-VA13 resulted in synthesis of a polypeptide with identical electrophoretic properties.

Selection of a stable cell line with constitutive expression of L-plastin To confirm L-plastin expression in these subclonal strains, the clonal cell number was expanded to obtain RNA for Northern blotting and to examine stability of L-plastin expression. Two of the Wi38-VA13 subclones, D-2.8 and C-2.3, grew in culture in a stable fashion. Hence, these clones were characterized in more detail, and the stability of L-plastin protein synthesis was examined.

L-plastin polypeptide synthesis was examined in the two stable Wi38-VA13 subclones which were expanded in cell number through at least 10 additional population doublings (the estimated number of doublings from a confluent 15-mm culture well to confluence in two 100 mm petri dishes). Subclone D-2.8 exhibited a low, but stable level of L-plastin synthesis, while subclone C-2.3 exhibited no trace of L-plastin expression like the parent Wi38-VA13 strain (Lin et al, *J. Biol. Chem.* **268**:2781-2792 (1993)).

A Wi38-VA13 G418-resistant subclone which was transfected with a cDNA gene placed under the control of the β -actin promoter was also analyzed. The electrophoretic position of

this recombinant form of L-plastin was identical to the electrophoretic species in D-2.8 tentatively identified as L-plastin (this can be judged by comparing the relative positions of polypeptides x and C with L-plastin). The 5 electrophoretic position of L-plastin in MRC5-SV2 (Lin et al, *J. Biol. Chem.* **268**:2781-2792 (1993), Celis et al, *Electrophoresis* **11**:1072-1113 (1990)), which was induced accompanying neoplastic transformation, was examined for comparison with the recombinant form and the induced form of 10 L-plastin in D-2.8.

The position of L-plastin in the MRC5-SV2 strain was also identical to the protein tentatively identified as L-plastin in D-2.8. However, the level of synthesis of L-plastin in D-2.8 was too low to be confirmed by Western blotting which 15 was used to confirm the identity of L-plastin in MRC5-SV2 (Lin et al, *J. Biol. Chem.* **268**:2781-2792 (1993)).

Northern blotting of RNA performed as described below was used to confirm the activation of the L-plastin gene in D-2.8, and its lack of expression in C-2.3.

20

Northern Blots Individual G418-resistant colonial strains which exhibited L-plastin expression in two dimensional gel protein profiles were expanded into mass culture for further analyses of RNA and genomic DNA as 25 described above. Total RNA was isolated by the guanidine isothionate, phenol-chloroform extraction method described by Chomczynski et al, *Anal. Biochem.* **162**: 156-159 (1987). Total cellular RNA (15 μ g) was electrophoresed on 1% agarose gels containing 2.2 M formaldehyde, and then blot transferred onto 30 nylon membrane. Northern blots were hybridized with L-plastin cDNA probe, a 476 bp HindIII fragment spanning codons 296 to 455 of the L-plastin cDNA (Lin et al, *Molec. Cell. Biol.*, **10**:1818-1821 (1990); Lin et al, *Mol. Cell. Biol.* **8**:4659-4668 (1988)), labeled with 32 P-dCTP by the random-priming technique 35 (Feinberg et al, *Anal. Biochem.* **137**:266-267 (1983)).

Blot 2 was a Northern blot analysis of L-plastin mRNA levels in HuLPPr-neo-selected subclones. The upper panel of

the blot was hybridized to the L-plastin specific probe and the lower panel was the same blot in which the L-plastin probe was stripped off and re-hybridized with a T-plastin probe (Lin et al, *Mol. Cell. Biol.* 8:4659-4668 (1988)). The sample RNA 5 of the blot was from the following cell lines:

	<u>Sample</u>	<u>Cell Line</u>
	1	HuT-13
	2	Wi38-VA13 subclone B-3.2
10	3	Wi38-VA13 subclone C-3.15
	4	Wi38-VA13 subclone C-3.10
	5	Wi38-VA13 subclone B-1.1
	6	Wi38-VA13 subclone C-2.3 cultured in the continuous presence of G418
15	7	Wi38-VA13 subclone C-2.3 cultured in the absence of G418 for one passage (approximately 3 population doublings)
	8	Wi38-VA13 subclone D-2.8 cultured in the continuous presence of G418
20	9	Wi38-VA13 subclone D-2.8 cultured in the absence of G418 for one passage (approximately 3 population doublings)
	10	Wi38-VA13 subclone D-2.1 cultured in the continuous presence of G418
25	11	Wi38-VA13 subclone D-2.1 cultured in the absence of G418 for one passage (this culture was dying and population doubling was not occurring)
30		

Blot 2 demonstrated the presence and elevation of L-plastin mRNA (3.7 kb) in D-2.8 grown in the absence or 35 continuous presence of G418 (Blot 2, samples 8 and 9, respectively) and the absence of detectable L-plastin mRNA in

C-2.3 also grown in the absence or continuous presence of G418 (Blot 2, samples 10 and 11, respectively).

The absence of L-plastin mRNA in C-2.3 was consistent with its failure to synthesize the L-plastin-like polypeptide 5 species. The level of L-plastin RNA in D-2.8 was lower than the level of L-plastin mRNA in HuT-13 fibrosarcoma cells that express L-plastin abundantly (Blot 2, sample 1; Leavitt et al, *Molec. Cell. Biol.* 6:2721-2726 (1986)). Even lower levels of L-plastin mRNA were detected in two additional subclonal 10 strains B-3.2 (Blot 2, sample 2) and C-3.10 (Blot 2, sample 4).

The exogenous L-plastin promoter fragments that had integrated into the genomic DNA of these Wi38-VA13 subclones were examined by Southern blotting using a DNA probe that 15 spanned the entire length of the genomic L-plastin promoter fragment to determine the amount of the exogenous L-plastin promoter DNA in the transfected strains.

Blot 3 was a Southern genomic blot performed as follows using a genomic HindIII fragment probe that was homologous and 20 hybridized to the 6 kb HindIII genomic fragment containing the L-plastin promoter (Lin et al, *J. Biol. Chem.* 268:2781-2792 (1993); Lin et al, *J. Biol. Chem.* 268:2793-2801 (1993)). For Southern blotting, 10 μ g HindIII digested genomic DNA (Feinberg et al, *Anal. Biochem.* 137:266-267 (1983)) was 25 electrophoresed on 0.7% agarose gels and hybridized with 6 kb HindIII L-plastin promoter DNA fragment isolated from L-plastin genomic clone pg305 (Lin et al, *J. Biol. Chem.* 268:2781-2792 (1993); Lin et al, *J. Biol. Chem.* 268:2793-2801 (1993)). Genomic DNA in the blot was from the following cell 30 lines:

	<u>Sample</u>	<u>Cell Line</u>
	1	Wi38-VA13
	2	Wi38-VA13 subclone B-3.4
35	3	Wi38-VA13 subclone D-2.1
	4	Wi38-VA13 subclone D-2.8
	5	Wi38-VA13 subclone C-2.3

While the parent Wi38-VA13 cell line exhibited only the expected 6 kb HindIII genomic hybrid (Blot 3, sample 1) corresponding to the endogenous gene, transfected subclones 5 exhibited the exogenous promoter sequence (a 5.1 kb EcoRI-HindIII promoter fragment) in the form of larger HindIII fragments.

D-2.8 (Blot 3, sample 4) exhibited approximately 2 diploid genomic equivalents of exogenous promoter DNA 10 determined by comparison of the hybridization signal of the larger exogenous restriction fragments with the intensity of the hybridization signal from the native genomic band (6 kb HindIII fragment). C-2.3 (Blot 3, sample 5) exhibited a more intense hybridization signal for the exogenous promoter 15 fragment suggesting the possibility of its amplification. Two other clones, B-3.4 and D-2.1 (Blot 3, samples 2 and 3, respectively) exhibited similar levels of exogenous L-plastin gene sequences (Blot 3, samples 2 and 5). Thus, the exogenous L-plastin promoter fragment was not further fragmented or 20 truncated during the transfection process.

The studies in Examples 7 and 8 indicate that most, if not all, neoplastic human cells exhibit some degree of activation of the L-plastin gene. In addition, this study demonstrates that a foreign gene, such as neo, attached to the 25 L-plastin promoter is expressed in cells having endogenous activation of the L-plastin gene.

WHAT IS CLAIMED IS:

1. A nucleotide sequence of not more than about 5.0 kilobases comprising a nucleotide sequence
5 corresponding to the sequence of the L-plastin promoter.
2. The nucleotide sequence of Claim 1 wherein said sequence corresponds to at least a portion of the L-plastin promoter upstream regulatory region.
- 10 3. An expression construct comprising a nucleotide sequence corresponding to the sequence of the L-plastin promoter and a foreign gene.
4. The expression construct of Claim 3 additionally comprising at least a portion of the L-plastin
15 upstream regulatory sequences.
5. The expression construct of Claim 4 wherein the upstream regulatory sequences include an estrogen responsive element.
6. The expression construct of Claim 4 wherein the
20 upstream regulatory sequences include a progesterone responsive element.
7. The expression construct of Claim 4 wherein the upstream regulatory sequences include at least about two kilobases of the upstream regulatory sequences.
- 25 8. The expression construct of Claim 7 wherein sequences corresponding to at least about five kilobases of the upstream regulatory sequences are included in the construct.
9. A method for inducing steroid responsive production
30 of RNA in a cell which is estrogen-responsive or progesterone-responsive comprising engineering said cell with an expression vector comprising a nucleotide sequence coding for said RNA sequence and a nucleotide sequence corresponding to the L-plastin
35 promoter and a portion of the upstream regulatory region containing a progesterone responsive element or the estrogen responsive element.

10. The method of Claim 9 wherein said nucleotide sequence coding for said RNA sequence is a DNA sequence encoding a protein.
- 5 11. The method of Claim 9 wherein said nucleotide sequence corresponding to the L-plastin promoter and a portion of the upstream regulatory region contains a progesterone responsive element and an estrogen responsive element.
- 10 12. A method for production of RNA in a hemopoietic cell comprising engineering said cell with an expression vector comprising a nucleotide sequence coding for said RNA sequence and a nucleotide sequence corresponding to the L-plastin promoter.
- 15 13. A method for determining whether an agent is cancer-causing comprising:
 - a. contacting a non-malignant tissue cell engineered with an expression vector comprising the L-plastin promoter and a reporter gene with said agent; and
 - 20 b. observing said cell for the expression of said reporter gene, the expression of said reporter gene indicating that the agent is cancer causing.
- 25 14. A method of expressing a gene encoding a cytotoxic product in cells that express L-plastin comprising transfecting said cells with a recombinant gene encoding a cytotoxic product under the control of the L-plastin promoter.
- 30 15. A method of selectively growing cells which activate the L-plastin gene comprising transfecting said cells with a recombinant selectable gene under the control of the L-plastin promoter.
16. The method of Claim 15 wherein the selectable gene is a drug resistance gene.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/00436

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12N 15/11, 15/63; C12P 19/34, 21/00, 21/02; C12Q 1/02

US CL : 435/29, 69.1, 70.1, 91.1, 320.1; 536/23.2, 24.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/29, 69.1, 70.1, 91.1, 172.1, 172.3, 240.2, 320.1; 536/23.2, 23.5, 23.7, 24.1

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,002,870 (LEAVITT et al.) 26 March 1991, see entire document.	1-16
Y	The Journal of Biological Chemistry, Volume 261, number 13, issued 05 May 1986, Epstein et al., "Isolation of a Rat Parvalbumin Gene and Full Length cDNA", pages 5886-5891, see especially pages 5888-5889.	1-16
Y	Proceedings of the National Academy of Sciences, Volume 84, issued July 1987, Gunning et al., "A human β -actin expression vector system directs high-level accumulation of antisense transcripts", pages 4831-4835, see especially pages 4832-4833.	1-16



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"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)	"&" document member of the same patent family
"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	

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INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 58, issued 1989, W. S. Dynan, "Modularity in Promoters and Enhancers", pages 1-4, see especially page 1.	5, 6, 9-11
Y	Biochimica et Biophysica Acta, Volume 951, issued 1988, B. Wasylyk, "Enhancers and transcription factors in the control of gene expression", pages 17-35, see especially page 18.	5, 6, 9-11
Y	Nature, Volume 301, issued 17 February 1983, Tavernier et al., "Deletion mapping of the inducible promoter of human IFN- β gene", pages 634-636, see entire article.	5, 6, 9-11
Y	Proceedings of the National Academy of Sciences, Volume 85, issued August 1988, van Zonneveld et al., "Type 1 plasminogen activator inhibitor gene: Functional analysis and glucocorticoid regulation of its promoter", pages 5525-5529, see especially pages 5527-5528.	5, 6, 9-11