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(54) Title: STABILIZED TRANSIENT GENE EXPRESSION

(57) Abstract

This invention provides methods and agents for enhancing transient expression in eukaryotic cells. Also provided are a model system for achieving prolonged transient expression in solid tumors, a means for culturing hepatocytes without feeder cells or an extracellular matrix bonded to the substratum, and a mehtod for manipulating cellular metabolism to reduce the cells' consumption of glucose.

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STABILIZED TRANSIENT GENE EXPRESSION

This application is a continuation-in-part based on U.S. Patent Application No. 08/833,747, filed April 11, 1997, which is a continuation-in-part based on U.S. Provisional Application 60/030,109, filed November 1, 1996.

Field of the Invention

This invention relates to methods and agents that enhance the transient expression of foreign genes that have been introduced into cultured eukaryotic cells.

Background of the Invention

The introduction of foreign DNA into eukaryotic host cells can serve many purposes. For example, this technique can provide a means of genetic complementation for identifying specific genes, e.g., a gene expressing an enzyme critical to a metabolic pathway can be identified by virtue of its ability to rescue cells defective in that pathway. Also, exogenous genes can be introduced for the purpose of exposing a recipient cell to a high dose of a protein not normally native to that cell, as for example, a cytotoxic protein introduced into a malignant cell for the purpose of killing it. Alternatively, foreign genes may be introduced into host cells to obtain the protein product of the foreign gene in sufficiently large amounts so that the protein can be harvested for further study or used as a pharmaceutical. In addition, the introduction of foreign genes is viewed as a promising avenue for somatic gene therapy. The goal of gene therapy is to cure inborn genetic defects by providing patients with a working copy of a missing or defective gene, or, alternatively, to provide a foreign gene product on a temporary basis for therapeutic purposes. One approach to somatic gene therapy is the *ex vivo* strategy, wherein cells are removed

from the body, transgenic DNA is inserted into the cells, and the cells are then returned to the body. In another approach, cells *in vivo* are targeted by foreign DNA that is introduced directly into the patient.

Foreign genes can be introduced into living eukaryotic cells by a variety of methods. These include, for example, the use of viral vectors into which the foreign gene has been ligated. Typically, viral vectors target only actively dividing cells (e.g., hematopoietic stem cells). Another method useful for the introduction of foreign DNA is electroporation, wherein cells take up DNA in response to a brief high voltage electrical pulse administered to the cells in the presence of a DNA solution. Other frequently used methods for introducing foreign DNA into cells include lipofection, in which the DNA is associated with liposomes, which are liquid-filled sacs formed by lipid molecules that aggregate to form a membrane structure. DNA molecules can become encapsulated in liposomes or can be associated with liposomal membranes. The liposomes are fused with recipient cells as a means of introducing foreign genes into the cells. Although widely used, one limitation of lipofection is that liposomes are somewhat toxic to living eukaryotic cells. In other commonly-used methods, DNA is co-precipitated with $CaPO_4$ before being applied to cells, or the entry of foreign DNA can be mediated by DEAE-dextran, a polymer that forms an electrostatic complex with DNA, the complex being internalized into cells by endocystosis (Kormis and Wu, *Seminars Liv. Dis.*, 15:257-267 (1995)). Exemplary transfection protocols are widely available (see, e.g., Sambrook et al., *Molecular Cloning*, 2d ed., (1989), which is hereby incorporated by reference; Gorman, C., "High Efficiency Gene Transfer into Mammalian Cells", Chap. 6, pp. 143-190 from *DNA Cloning II - A Practical Approach*, IRL Press, Oxford (1985), Ed. Glover, D.M.; Wynshaw-Boris et al., *BioTechniques*, 4:104-119 (1986); Chang, P.L. (Ed), *Somatic Gene Therapy*, CRC Press, 1995; and *Guide to Eukaryotic Transfection with Cationic Lipid Reagents*, Life Technologies (Gibco-BRL)).

"Transfection" is a general term often used to describe processes by which foreign genes ("transgenes") are introduced into a living host cell. Another term for this process is "transduction," this latter term being most commonly used when referring to viral vector-mediated gene transfer. Host cells that express or incorporate the foreign DNA are known as "transformed cells," and the process by which they become transformed is called "transformation" or "transduction." Different types of cells vary in their susceptibility to transformation, and protocols for introducing the foreign DNA are typically optimized by the adjustment of various parameters such as

pH, type of culture medium, amount of DNA, CO_2 concentration, or method of DNA introduction (see, e.g., Chen and Okayama, *Mol. Cell. Biol.*, 7:2745-2752 (1987)).

When foreign DNA is introduced into cells by the $CaPO_4$ or by the DEAE-dextran method, it has been observed most of the cells initially take up the DNA, but only a fraction of them will express the DNA during the first few days after it has been introduced (see, e.g., Gorman (1985)). Early expression of transfected DNA is typically short-lived, and is referred to as "transient expression." A still smaller fraction of the recipient cells (0.1-0.001%) will stably incorporate the transfected DNA by covalently linking it into the host genome (see, e.g., Wynshaw-Boris et al. (1986)). When transfected foreign DNA becomes covalently inserted into the host DNA in a fashion that supports expression of the foreign gene, the resulting cells are said to be "stably transformed." A likely reason for the observed low level of covalent integration is that active DNA synthesis must occur in order for integration of the foreign DNA to take place. Thus, cells are considered to be susceptible to stable transformation only during the S phase of the cell cycle.

In stably transformed cells, the foreign gene is reproduced each time the cell divides, and the protein encoded by the foreign gene is expressed by the same enzymatic machinery that transcribes the endogenous cellular chromosomal genes. However, after the initial wave of transient expression, only a tiny fraction of the cells in a transfected culture typically contain integrated copies of the transfected gene. Thus, the amount of foreign protein expressed in such a culture will be small, and may even be undetectable. Accordingly, stable transformation protocols generally rely on a post-transfection selection step to provide a selective growth advantage to the few stably transformed cells that are present following the addition of foreign DNA.

Homogenous cultures of stably transformed cells can be selectively isolated under a variety of experimental conditions to obtain lines of cells that have integrated the foreign gene, and that continue to express it. To accomplish this, a selectable gene, usually one that confers drug resistance or that encodes a chromogenic protein, must be introduced into the host cell concurrently with the introduction of the DNA encoding the desired protein. Examples of reporter genes suitable for this purpose include bacterial chloramphenicol acetyltransferase, luciferase, alkaline phosphatase, bacterial β-galactosidase, and others (see, e.g., Alam and Cook, *Anal. Biochem.*, 188:245-254 (1990)). If a drug-resistance marker is being used, drug resistant cells must be selected by long-term exposure to the relevant drug in order that the stably transformed cells can become predominant in the culture. Alternatively, cells

containing integrated DNA may be identified by their expression of a co-transfected gene that is capable of converting a chromogenic substrate into a colored substance that permits the identification and manual cloning of individual stably transformed cells. In some instances, the desired gene itself may confer a selectable trait on the stably transformed cells, but this circumstance is rare. In any case, the creation and isolation of stably transformed cell lines can take one to three months to accomplish. (Wynshaw-Boris et al. (1986)).

In contrast to stable transformation, the transient expression of transfected DNA does not depend on the integration of the foreign DNA into host cell chromosomes. Although the majority of DNA applied to a cell is believed to be rapidly transported into the nucleus, in some systems expression can be detected for up to 80 hours post-transfection in the absence of any detectable integration (see, e.g., Gorman (1985)); Wynshaw-Boris et al. (1986)). No selection step is required before transient expression can be detected. However, only about 1-10% of cells that take up foreign DNA typically transcribe mRNA from a transfected foreign gene (see, e.g., Gorman et al., *Nucl. Ac. Res.*, 11:7631-7648 (1983)). Although the vast majority of transfected DNA in transiently transfected cells does not become incorporated into the host DNA, it does become incorporated in about 0.001-1% of these cells (Alam and Cook (1990)). This small stably transfected fraction of cells is believed to play no significant or useful role in the foreign gene expression profile observed immediately after transfection.

Without a selection step, the expression of foreign genes quickly disappears from cultures of transfected cells. Typically, transient expression in cultured cells peaks in about 48 hours, and is detectable for only 24-80 hours. (Gorman (1985); Wynshaw-Boris et al. (1986)). It is widely believed that most of the DNA taken up by transfected cells becomes rapidly catabolized by nucleases or becomes diluted by cell division (see, e.g., Gorman (1985); *Guide to Eukaryotic Transfection with Cationic Lipid Reagents Life Technologies*).

Because transient expression does not require that the target cells are actively dividing, it can be achieved in terminally differentiated cells that do not normally divide, although susceptibility to transfection varies dramatically among such cells. For example, naked DNA can be expressed when injected directly into mouse skeletal muscle (Wolff, et al., *Science*, 247, 1465-1468 (1990)). In other studies, naked DNA was used as a vaccine (Cohen, J., *Science*, 259, 1691-1692 (1993)).

Many studies have focused on the liposomal delivery of foreign DNA *in vivo* to hepatocytes (see, e.g., Wu and Wu, *J. Biol. Chem.* 263:14621-14624 (1988); Chow et al., *J. Pharmacol. Exp. Ther.*, 248:506-13 (1989); Wu et al., *J. Biol. Chem.*, 264:16985-16987 (1989); Kaneda et al., *J. Biol. Chem.*, 264:12126-12129 (1989a); Kaneda et al., *Science*, 243:375-378 (1989b); Wilson et al., *J. Biol. Chem.*, 267:963-967 (1992); Wilson et al., *J. Biol. Chem.*, 267:11483-11489 (1992b); Chowdhury et al., *J. Biol. Chem.*, 268:11265-11271 (1993); Perales et al., *Proc. Natl. Acad. Sci. USA*, 91:4086-4090 (1994); Kormis and Wu, (1995)). One approach to targeting foreign DNA to specific tissues *in vivo* is receptor-mediated liposomal delivery (reviewed in Kormis and Wu (1995)). In applying this strategy to liver, Wu and his colleagues exploited the presence of asialoglycoprotein receptors on hepatocyte surfaces to target injected liposomes to the liver. This delivery system is characterized in a series of publications (Wu and Wu (1988); Wu et al. (1989); Wilson et al. (1992a); Wilson et al. (1992b); Chowdhury et al. (1993); Perales et al. (1994)). The asialoglycoprotein was packaged into liposomes together with DNA that had formed an electrostatic complex with polylysine. When initial efforts were successful, this group attempted to maximize the stable integration of the foreign DNA by performing partial hepatectomies in the recipient rats. As regenerating liver cells provide a higher proportion of cells in S phase than are present in normal liver, this tactic was expected to increase the proportion of liver cells into which foreign DNA could integrate. After partial hepatectomy, the transgenic protein was detectable in the blood for as long as 11 weeks post-transfection (Wu et al. (1989)). At first, these investigators believed that the injected DNA had become integrated, but later experiments revealed no detectable integrated DNA, showing instead that the preserved foreign DNA resided in the plasma membrane/endosome fraction (Wilson et al. (1992b); Chowdhury et al. (1993)). This surprising observation indicated that partial hepatectomy leads to the persistence of transgenic DNA by a mechanism that is independent of DNA synthesis *per se*.

Another group also has employed a targeting strategy for directing injected DNA to the liver (Kaneda et al. (1989a); Kaneda et al. (1989b)). Here, transgenic DNA was packaged in liposomes with proteins normally found in the nucleus, i.e., the non-histone chromosomal proteins. They observed transport of the injected vesicles to the nuclei of liver cells, and detected measurable transgene expression for up to 7 or 8 days after injection. However, this DNA did not become integrated into the liver cell chromosomes. Others have reported the successful *in vivo* expression of foreign

DNA following the injection of $CaPO_4$-DNA precipitates directly into the liver, spleen, or peritoneum (see Kaneda et al. (1989a)).

A number of reagents have been shown to increase the efficiency *in vitro* of stable transformation. One group has reported that by controlling the pH in the culture medium during $CaPO_4$ mediated transfection, stable transformation efficiencies as high as 50% can be achieved (Chen and Okayama (1987)).

Another reagent reported to enhance the expression of transfected DNA is butyric acid or its sodium salt (Gorman et al. (1983)). Because of its known ability to alter chromatin structure, Gorman et al. (1983) tested the effects of butyric acid on the expression of foreign genes introduced into cells by transfection. In these studies, cells were transfected, then exposed to a single 12 hour dose of sodium butyrate. When transient expression was monitored over the next five days, they observed a 2-4-fold increase in the percentage of recipient cells that expressed the transfected gene. They also noted 25-100-fold increases in the foreign gene expression levels when the transfected construct included an SV40 enhancer element. When other cultures transfected in the presence of butyrate were selected for stable transformants, they observed a significant increase over controls in the percentage of transfected cells that gave rise to stable transformants. However, Palermo et al. (*J. Biotech.*, 19:35-48 (1991)) found that butyrate could induce increased transgene expression in stable transformants whether or not butyrate had been present during the transfection step. Indeed, many reports have documented butyrate's ability to induce the synthesis of certain proteins or to increase cell differentiation *in vitro*. (Boffa, et al., *J. Biol. Chem.*, 256:9612-9621 (1981); Kruh, *Mol. Cell. Biochem.* 42:65-82 (1982); Chabanas, et al., *J. Mol. Biol.*, 183:141-151 (1985); Parker, *J. Biol. Chem.*, 261:2786-2790 (1986); Kooistra, et al., *Biochem. J.*, 247:605-612 (1987); Kaneko, et al., *Canc. Res.*, 50:3101-3105 (1990); Nathan, et al., *Exp. Cell Res.*, 190:76-84 (1990); Palermo, *J. Biotech.*, 19:35-48 (1991); Kosaka, et al., *Exp. Cell Res.*, 192:46-51 (1991); and Oh, et al. *Biotechnol. Bioeng.*, 42:601-610 (1993)). Optimal concentrations of butyrate for gene induction vary from cell type to cell type, and a suitable concentration range that minimizes its cytotoxic effects must be empirically determined for each type of target cell (see, e.g., Gorman (1985); Parker et al. (1986); Oh et al. (1993)).

The effects in living cells of butyric acid (or butyrate) are not limited to the modification of chromosome proteins. One of this compound's most striking manifestations is its ability to reversibly suppress the growth of cultured cells (see,

e.g., Boffa et al (1981), reporting that most cells exposed to butyrate are arrested at the G1/S boundary). Moreover, butyrate enhances the antitumor action of interferon (Kruh (1982)). This antitumor activity is particularly interesting, as it was achieved by injecting 0.5 ml of a 50 mM butyrate solution directly into live mice.

There are many advantages to using transient expression rather than stable transformation for the expression of foreign genes. First, by using transient expression, one can quickly analyze a relatively large number of constructs. Also, it may be the method of choice for delivering therapeutic proteins whose presence in the body is desired only for the duration of the disease. Furthermore, transient expression avoids the danger of mutagenesis or cell death that may occur as the result of foreign DNA being inserted into a critical cell gene. In addition, transient expression can be achieved in primary cell lines that are not immortalized, whereas stable transformants can be established only from cells that can survive and divide in cultures for long periods of time. However, with the exception of the liver hepatectomy model, a major drawback of currently known transient expression methods continues to be the relatively short-lived expression of the foreign gene, and the tendency of transfection reagents, including purified DNA itself, to be toxic to living cells. Hepatectomy or other surgical excisions are too drastic an approach for most practical purposes. Thus, the availability of other means of stabilizing transient expression would widen the potential range of applications for this technique.

Summary of the Invention

This invention provides methods and agents that significantly enhance the expression of foreign DNA that has been introduced into a host eukaryotic cell. The agents described herein increase both the amount and duration of transient expression. The chemical compounds that comprise these agents are demonstrated to be efficacious in both growing cells and in static cultures of non-dividing cells. This circumstance indicates that the enhanced transgenic expression observed with these compounds does not involve integration of the foreign DNA into the genome of the recipient host cell.

Furthermore, it is shown that the compounds of this invention depress the consumption by cultured cells of glucose present in the culture medium, thus forcing the cells to rely for energy on alternative carbon sources, such as lipids or proteins. These same cells exhibit increased production of ammonia, thus suggesting that protein is being used as a source of energy. Their effect on glucose consumption suggests that the principal site of action of these compounds is the mitochondria. This

observation implicates the mitochondria as playing a critical role in sustained expression of non-integrated foreign DNA both *in vitro* and *in vivo*. In addition, cells grown in the presence of these compounds become induced to express and secrete an endogenous alkaline phosphatase activity.

The invention further provides long-term transient expression of foreign genes that have been introduced into target cells by a variety of delivery systems, including but not limited to cationic lipids (i.e., liposomes) and various synthetic polymers such as dendrimers (also known as "starburst" polymers). Many of the subject chemical compounds influence the fate of foreign gene expression well after the foreign DNA has been introduced into the cell, thus act independently of the method by which the DNA is introduced. Some of the subject compounds are especially effective in increasing the degree of expression during the first four days following the introduction of foreign DNA, thus appear to enhance the initial amount of DNA taken into the cells, or to increase the proportion of cells that express the DNA, or both.

The compounds of this invention have a hydrophobic moiety and an acidic moiety, and the latter may take the form of a salt or an ester. Moreover, they are biocompatible, i.e., when applied to cells at appropriate concentrations, greater than 50% of the cells remain viable.

The methods of the subject invention involve categorizing the compounds into "Type A" formulations, which primarily increase the degree of transient expression during the first four days after foreign DNA is added to the cells, and "Type B" formulations, which primarily stabilize transient expression after foreign DNA has entered the cell. Thus, the designations "Type A" and "Type B" reflect *when* the compounds are added to the cultures. Optimal expression is obtained by treating cells with a Type A compound before, during, and/or after the transfection step (first phase of transient expression), and by further adding a Type B compound within hours or days (e.g., within 12-60 hours) of introducing the foreign DNA, and leaving it in contact with the cells thereafter (second phase of transient expression). Optimally, the Type A compound is maintained in the medium throughout both phases of transient expression. The invention further provides an assay for determining the efficacy of individual chemical compounds and formulations of two or more compounds for their use in both phases of transient expression.

The chemical compounds of the subject invention create profound metabolic changes in living cells, resulting in their being able to sustain the transient expression of foreign DNA for periods far longer than previously observed. Moreover, following

the addition of these chemical substances, cultured cells surprisingly reduce their consumption of glucose, and concomitantly increase their use of alternative energy sources, such as proteins and possibly lipids.

In other embodiments, this invention provides methods for culturing hepatocytes in the absence of feeder cells and without the need to pre-coat cell culture substrata with proteinaceous or other adhesion-promoting molecules.

Brief Description of the Drawings

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same becomes better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIGURE 1 shows a cell growth curve for cells exposed to several different compounds of the subject invention. FIGURE 1 graphically depicts the amount of cell growth for some of the plates described in Example 4 and Table 7. The numbers in the inset boxes of FIGURE 1 correspond to the plate numbers listed in Table 7;

FIGURE 2 graphically illustrates the cytoxicity of some of the compounds whose test results are presented in Table 7. The numbers in the inset boxes of FIGURE 2 correspond to the plate numbers listed in Table 7; and

FIGURE 3 graphically illustrates the results of the experiments of Example 6. These experiments involved transient expression in differentiated porcine PICM-19 3BT cells, which resemble hepatocytes, in the presence of various chemical compounds that prolong the duration of transient expression.

FIGURE 4 is a graphic illustration of the amounts of β-galactosidase measured in the samples harvested daily during the experiment described in Example 8, and illustrates the long-term stabilization (i.e., 32 days) of transgene expression in transfected cells cultured in a bioreactor device in the presence of transient expression-stabilizing compounds.

FIGURES 5A-5C graphically illustrate the concentrations of ammonia, glucose, and lactate in the culture medium sampled daily during the experiment described in Example 8. FIGURE 5A indicates the concentration of ammonia measured in each sample; FIGURE 5B indicates the concentration of glucose measured in each sample; and FIGURE 5C indicates the concentration of lactate measured in each sample.

Detailed Description of the Preferred Embodiment

Definitions:

Transfection: For purposes of the following disclosure, "transfection" will refer to any means of introducing foreign DNA into a recipient cell, including liposome-mediated methods, viral vectors, $CaPO_4$-DNA coprecipitates, DEAE-dextran, naked DNA, DNA complexed with proteins transfection in the presence of starburst polymers, or other means of introducing the DNA into the recipient cell.

Foreign DNA/transgenic DNA: Genetic material that has been appropriately modified for expression in recipient eukaryotic cells, typically but not necessarily originating from an organism other than the recipient cell. Transgenic DNA typically will contain the coding region for a biologically active protein or protein domain. By "appropriate modifications," it is meant that the transgenic DNA is operably linked with a eukaryotic promoter and any other regulatory sequences required to ensure functional transcription of the foreign gene in the host cell. The DNA usually is in circular form, and contains the coding region for a polypeptide operably linked to the regulatory signals necessary for transcription and the subsequent translation of the resulting mRNA. Such signals may include a promoter for binding RNA polymerase, an enhancer, transcription termination signals, ribosome binding sites, translation start and stop signals, poly(A) addition signals, and so on. The enhancer may be tissue-specific.

β-galactosidase (β-gal): A bacterial enzyme able to convert a colorless substrate into an easily detectable colored product. This gene is used in the Examples described below as a representative foreign gene for the purposes of demonstrating the efficacy of this invention.

The subject invention provides methods and agents for enhancing the transient expression of foreign genes in eukaryotic cells. The methods have been shown to be efficacious in human colon carcinoma cells, mouse melanoma cells, porcine primary hepatocytes, and in a porcine cell line that resembles differentiated hepatocytes. This method involves first introducing into the cell a molecule of foreign DNA that encodes a protein in a form capable of being expressed in the cell.

The foreign DNA may be introduced into the cell by any convenient method, including but not limited to, lipofection, electroporation, incubation with $CaPO_4$-DNA coprecipitates, incubation with DEAE-dextran, ligation of the DNA into a viral vector, and so on. Vectors derived from retroviruses or adenoviruses are useful for introducing foreign DNA into eukaryotic host cells. If desired, the plasmid or viral

vector containing the foreign DNA may provide nucleotide sequences positioned between the promoter and the insertion site, or alternatively, positioned following the insertion site, such that one or more amino acids encoded by the vector-provided nucleotide sequences become fused to the protein encoded by the foreign DNA. Such fusion sequences can provide peptides that direct desired post-translational modifications, such as signal peptides for secretion, or sites for attachment of carbohydrate moieties.

The subject invention provides methods and agents for enhancing the transient expression of a foreign gene in a cell. Before, during, or after the introduction of the foreign DNA into the cell, the cell is contacted with one or more of the chemical compounds described below, whereafter the expression of the foreign DNA becomes substantially enhanced as compared with cells transfected in the absence of these compounds. By "enhancing transient expression," it is meant here that when the subject methods are used, the amount of transgene expression during the first few days following transfection is increased as compared with controls, or that the period during which transient expression occurs is prolonged as compared with controls, or both. In the subject methods, cells into which foreign DNA is introduced are contacted with a chemical agent that increases the efficiency of initial DNA uptake or expression, or that prolongs the effective half-life of the foreign DNA after it has entered the cell. Individual compounds may increase the efficiency of initial expression, and also prolong the period of transient expression. The term "effective half-life of the foreign DNA" is used here in a chronological sense to refer to the length of the period during which protein encoded by the foreign DNA can be detected in the cells. When conventional transfection protocols are used, i.e., when transfection is performed without using the below-described chemical compounds according to the disclosed methods, transient expression typically decays to an undetectable level within 3-4 days after the foreign DNA has been introduced into the cells. However, using the subject methods, easily detectable levels of expression are typically observed as long as 14 days after the transfection step, and have been observed for as long as 32 days after transfection. DNA and RNA homologous to the transfected foreign DNA has been detected 32 days post-transfection in transfected cells that were treated according to the subject methods. Using the subject methods, transient expression typically peaks within about 2-3 days, then drops off to a level of about one-third the initial level, thereafter remaining stable for several days to several weeks.

Agents useful for the subject methods include a large number of chemical compounds that are described more fully below. An "agent" may consist of a single chemical compound, or a combination of two or more compounds. Moreover, the agent may include one or more compounds administered during the early phases of the transfection protocol, and an additional compound or compounds added after the foreign DNA has entered the cell. These transient expression enhancing agents may be present before, during, and after the introduction of foreign DNA. When added to the cells after introducing the DNA, the agent typically remains in contact with the cell for at least 24 hours, or longer.

Generally, chemical compounds useful as agents in the subject method include at least one hydrophobic moiety and at least one acidic moiety. The acidic moiety may also be hydrophobic and organic. For certain of these chemical compounds, the acidic moiety has been modified as a salt or an ester. In one embodiment, the chemical compounds are carboxylic acid derivatives represented by the general formula R_1-C(=O)-OR_2, and in another embodiment, the chemical compounds are sulfonic acid derivatives represented by the general formula R_7-SO_2-OR_8.

Suitable carboxylic acid derivatives (i.e., R_1-C(=O)-OR_2) include naturally occurring amino acids (e.g., glycine, alanine, valine, leucine, isoleucine, aspartic acid, glutamic acid, glutamine, serine, threonine, methionine, arginine, lysine, histidine, proline, tryptophan, phenylalanine, tyrosine), their unnatural optical isomers, and certain amino acid derivatives (e.g., 3-methyl-L-histidine, α-ketoglutaric acid, β-alanine, carnosine, citrulline, creatine, folic acid, glutathione, hippuric acid, homoserine, N-carbamyl aspartic acid, N-formyl-L-methionine, and ornithine).

Referring to the general carboxylic acid derivative formula, R_1-C(=O)-OR_2, for the amino acids, R_1 is $CHNH_2R_3$, wherein R_3 is the side chain of a naturally occurring amino acid. Other amino acid derivatives that are useful in the method of the present invention include amino acids that further include alkyl substituents and alkyl substituents having additional functional groups. These amino acid derivatives are represented by the above carboxylic acid derivative formula where R_1 is -$CHNH_2(CH_2)_nR_5$, wherein n = 1-7 and R_5 is selected from CH_3, OH, $CONH_2$, C_6H_4OH, and $CONHNH_2$. Alternatively, R_1 is -$(CH_2)_nCHNH_2CO_2H$, wherein n = 1-8; -$CH(CO_2H)NHCONH_2$, or R_1 is -C_5H_4N (i.e., nicotinic acid and derivatives).

In addition to amino acids, the carboxylic acid derivatives useful in the present method include alkyl, aryl, and substituted alkyl and aryl carboxylic acid derivatives. Preferred alkyl and substituted alkyl carboxylic acid derivatives are represented by the

general formula above where R_1 is $-(CH_2)_nR_6$, wherein n = 1-9 and R_6 is selected from an indole group, $NCH_3C(=NH)NH_2$, SCH_3, NH_2, CH_3, CO_2H, $CONH_2$, and $NHC(=NH)NH_2$. Preferable aryl carboxylic acid derivatives include benzoic acid and its derivatives. The benzoic acid derivatives are represented by the formula above where R_1 is $-C_6H_4R_4$, wherein R_4 is selected from H, CH_3, $(CH_2)_nCH_3$, NH_2, $COCH_3$, $CO(CH_2)_nCH_3$, $C(CH_3)_3$, $CH(CH_3)_2$, $(CH_2)_nCH(CH_3)_2$, $(CH_2)_nCOCH_3$, OCH_3, and $O(CH_2)_nCH_3$, wherein n = 1-3. It has been found that branched chains often are more efficacious than linear drains.

The carboxylic acid derivatives useful in the present invention include carboxylic acids (i.e., R_2 is H); carboxylic acid esters (e.g., R_2 is CH_3 and $(CH_2)_nCH_3$ wherein n = 1-8) including esters having additional functional groups such as ether and ketone groups (e.g., R_2 is $(CH_2)_xO(CH_2)_yCH_3$ and $(CH_2)_xCO(CH_2)_yCH_3$ wherein x + y = 2-7); and carboxylate salts including metallic salts (e.g., lithium, sodium, potassium, calcium, and magnesium) as well as relatively low molecular weight cations (e.g., ammonium).

Suitable sulfonic acid derivatives, represented by the general formula $R_7\text{-}SO_2\text{-}OR_8$, include alkyl, aryl, substituted alkyl and aryl sulfonic acid derivatives (i.e., R_7 is an alkyl, aryl, or substituted alkyl or aryl group). Preferably, the sulfonic acid derivative is a lower alkyl (i.e., straight chain or branched C_1-C_5 alkyl group) sulfonate, and more preferably, an amino substituted lower alkyl sulfonate, for example, taurine. Preferably, the aryl sulfonic acid derivative is a benzene sulfonic acid derivative, and more preferably, an amino substituted benzene sulfonate, for example, 3-aminobenzene sulfonic acid. The sulfonic acid derivatives useful in the present invention include sulfonic acids (i.e., R_8 is H), and sulfonic acid salts including metallic salts (e.g., R_8 is lithium, sodium, potassium, calcium, or magnesium) as well as relatively low molecular weight organic cations (e.g., R_8 is ammonium ion).

In another embodiment, the chemical compounds useful as agents in the subject methods include sulfonated amino polysaccharides. Preferably, the polysaccharide is a sulfonated N-acetylated amino polysaccharide. Suitable polysaccharides typically contain from about 4 to about 200 sugar residues.

Preferred sulfonated N-acetylated amino polysaccharides include chondroitin-6-sulfate and sulfonated guarans. Guarans are isolated from the endosperm of *Cyamopsis tetragonolobus* seeds and are high molecular weight β-1,4 D-galactomannans (e.g., up to about 1,200,000 daltons) having α-1,6-linked D-

galactose residues attached to a mannan backbone. Suitable guarans include 2-hydroxypropyl ether derivatives commonly referred to as hydroxypropyl guarans.

Other chemicals compounds that are useful in the method of the present invention include adrenaline (epinephrine), coenzyme B12, and methylcobalamin.

An especially effective example of a sulfonated amino polysaccharide identified thus far is chondroitin-6-sulfate (type C chondroitin sulfate), a polyanionic glycosaminogycan. Chondroitin sulfate is a naturally-occurring mucopolysaccharide found in cartilage and soft connective tissue throughout the body. Type C chondroitin sulfate is a polymeric molecule that varies in its molecular weight, degree of sulfonation at the N-acetylgalactosamine residue of the repeat disaccharide, and in the relative distribution of sulfated to unsulfated repeat units. Chondroitin-6-sulfate having a molecular weight of about 4000 daltons is particularly effective. Commercial preparations of type C chondroitin-6-sulfate typically contain a variable proportion of type A chondroitin sulfate, which is chondroitin-4-sulfate. While preparations containing predominantly type C chondroitin sulfate are highly effective for stabilizing transient expression, preparations that contain predominantly the type A form of this polymer are ineffective. Thus, the relative amount of 4-sulfate to 6-sulfate substitution sites occupying the N-acetylgalactosamine residue of the repeat disaccharide in a given polymer preparation is critical to the polymer's activity in the subject method, as only the 6-sulfate form appears to be active in enhancing transient expression.

Chemical compounds effective in enhancing transient expression have the following desirable characteristics in common:

1. Little or no cytotoxicity when added to cells in culture within concentration ranges effective for enhancing transient expression. For the sulfonated amino polysaccharides, this range is about 0.01-0.5 mM. For the remaining compounds effective in enhancing transient expression, this range is about between 1-15 mM. These optimal amounts are in addition to quantities of these substances that may already be present as a cell culture media component (e.g., certain amino acids). Compounds that are not cytotoxic according to this assay are defined here as "biocompatible." For the purposes of this invention, a cytotoxic substance may be defined as one that, at a given concentration, results in >50% decline in the number of viable cells within 4 days post-transfection in an 8 day static culture of SW480 cells, with continuous exposure to the substance, and wherein no net expansion of the cells occurs by the end of the 8 day period. However, it should be understood that various

types of cells vary in their sensitivity to different chemical compounds. Thus, while SW480 cells may be used as a convenient tool for determining biocompatible concentrations of the chemical compounds, it may be necessary to empirically adjust the concentrations determined with SW480 cells in order to optimize biocompatibility with other types of cells. Assays for cytotoxicity are described in greater detail in Example 4.

2. An acidic functional group is always present (e.g., carboxylic, sulfonic, and the like), and may be modified to reduce cytotoxicity. Preferred modifications are ester and salt formation (including salt based on organic cations), as salt and ester bonds are readily cleaved by metabolic processes after the compound has entered the target cell. In a preferred embodiment, the chemical compound in aqueous solution has a pH of 4.5-9.0.

3. To the acidic group is linked a relatively hydrophobic organic group. For compounds other than the sulfonated polysaccharides, this portion of the molecule is preferably non-polar and hydrophobic. Similarly, the sulfonated polysaccharides have their natural hydrophilic character modified by the presence of a relatively hydrophobic functional group (e.g., the N-acetyl group in chondroitin-6-sulfate in the 2-substitution position). Many of the compounds that have proven efficacious for the subject invention contain an acidic group that is organic and hydrophobic.

4. Several of the most effective agents (e.g., a 50/50 mixture of benzoic acid and sodium benzoate (benzoate buffer), and chondroitin-6-sulfate) possess antioxidant and free radical scavenging character (e.g., see Merck Index).

For convenience, the compounds other than the sulfonated amino polysaccharides will hereafter be referred to as "Group I," while the sulfonated amino polysaccharides will hereafter be called "Group II." In a preferred embodiment of the invention, the cells are contacted with a chemical compound selected from Group I prior to and during the introduction into the cell of the foreign DNA, and are further contacted with a chemical compound selected from Group II following the introduction of the foreign DNA. The chemical compounds of the subject invention are efficacious whether they are added to the cells prior to, during, or after the transfection step.

The subject invention provides methods useful for prolonging transient expression in cultured cells, including primary cultures, established cell lines, stable cultures of differentiated cells, normal cell lines maintained by exposure to growth

factors, and transformed cells, such as cultures established from various tumors, including hybridoma cells and the SW480 P3 human colon carcinoma cell line (ATCC #CCL228; hereafter referred to as "SW480 cells").

The subject methods are useful also for introducing foreign DNA into cells *in vivo*. The compounds can be administered by any convenient means, including orally, topically, by perfusion, or by injection. If it is desired to confine the host's exposure to the chemical compound to the tissues that will receive foreign DNA, the chemical compound can be introduced by localized injection, such as, for example, injection directly into a solid tumor mass, or by incorporating into liposomes a protein that targets them to specific tissues. Injections of the chemical or combination of chemicals can be accompanied by or followed by injection at the same site of a vehicle for delivering the foreign DNA. Alternatively, the compounds can be applied in a vehicle that provides for the slow release of the compounds at the target site, as, for example, by the dissolution of an inert solid carrier.

The method of the subject invention contemplates the harvesting of proteins expressed by transgenic DNA introduced into cells in the presence of the above-described compounds. The protein can be harvested by any convenient means, such as, for example, by extracting the transfected cells, or by extracting the culture medium in which the cells are grown. If desired, the transgenic protein can be expressed using a vector that provides signal peptides that direct the secretion of the transgenic protein into the culture medium. Secreted protein also can be assayed over a period of days to determine the relative or absolute amount of protein produced, thus providing a means for evaluating the effectiveness of variations in the transfection protocol. Crude cell extracts can be assayed for enzymatic or other biological activity of the harvested protein, or the protein can be further purified using standard procedures before performing functional assays for the protein's activity. If the transgene is expressed *in vivo*, the protein can be harvested from body fluids of the host, such as milk, or other body tissues. The purification procedure used for a given protein will depend on the physical properties of the protein, such as its size, shape, hydrophobicity, stability, and so on. Also, the harvested protein may be detected or quantified by physical means, such as, for example, gel electrophoresis, isoelectric focusing, or by chromatographic methods such as high-pressure liquid chromatography, or the like.

The methods of the subject invention result in the rapid production in cultured eukaryotic cells or in transiently expressing mammalian hosts of milligram quantities

of the protein product of the expressed transfected genes. Protein production in transfected eukaryotic cells provides an advantage over bacterial expression systems in that expression in eukaryotic cells can support post-translational modifications that may be required for the biological activity of many proteins. Moreover, by using eukaryotic instead of prokaryotic host cells, one avoids having to eliminate potentially toxic bacterial proteins from final preparations of the transgenic protein.

The subject methods provide a means for using eukaryotic host cells to obtain commercially useful amounts of biologically active protein, e.g., growth factors, hormones, antibiotics, and the like, in a eukaryotic host cell without having to establish permanent cell lines containing stably integrated foreign DNA. These methods can be used for rapidly obtaining biologicals to be tested for their pharmaceutical properties.

Contacting cells with the chemical compounds selected from Group II of the subject invention results in increased cell adhesion and cell-to-cell contact and communication, thus administration of these compounds provides a means for enhancing these cell-cell interactions. Thus, the subject invention includes methods for enhancing the adhesion of a cell to a culture substratum by growing the cells in the presence of a sulfonated amino polysaccharide that has been added to the culture medium, thus promoting the longevity of the cells in culture. For example, chondroitin-6-sulfate is effective in promoting the long-term growth in culture of a cell line that has the characteristics of differentiated hepatocytes. Ordinarily, hepatocytes will not survive in culture unless feeder cells are provided or the culture substratum is first coated with a substance to promote hepatocyte adhesion (see, e.g., Sidhu and Omiecinski, *Pharmacogenetics*, 5:24-36 (1995)).

When agents of the subject invention were contacted with cultured cells, the cells exhibited altered metabolic processes, including reduced glucose consumption and lactate production, as well as increased ammonia production. Thus, the subject methods are useful for manipulating the metabolism of a cell such that the cell utilizes alternative carbon sources such as proteins or lipids. In addition, the agents induce cells to express elevated levels of an endogenous alkaline phosphatase activity. Compounds of both Groups I and II are useful for this purpose. An agent especially useful for manipulating a cell's utilization of energy sources is a combination of benzoic acid, 4-ethylbenzoic acid, chondroitin-6-sulfate, and benzoate buffer, wherein benzoate buffer is an equimolar mixture of benzoic acid and sodium benzoate. The

subject methods are useful for manipulating cell metabolism either *in vitro* or *in vivo*, e.g., to treat mammals for obesity.

Because of their hydrophobic nature, the chemical compounds of the described methods may be capable of crossing the highly hydrophobic mitochondrial outer membrane. As these agents affect mitochondrial-based metabolic processes, i.e., glucose metabolism, it seems possible that the observed enhanced expression results from the transcription of the foreign DNA inside of mitochondria. As the foreign DNA is circular, it may even be replicated by the machinery normally used for replicating the circular mitochondrial DNA, thus increasing the amount of template available for expressing the transgene.

The subject invention provides for the use of these chemical compounds as agents for enhancing transient expression both *in vitro* and *in vivo*. When employed either *in vitro* or *in vivo*, the compounds are optimally used before, during, and after the introduction of foreign DNA. On removal of the chemical compounds from the cell culture media, their effect on long-term expression gradually disappears and the previous behavior of the cell resumes. When employed *in vivo*, the compounds may be injected as a primer into the recipient tissue or intravenously admixed with the transgenic DNA solution, and administered after introduction of the foreign gene by injection, or may be administered as a dietary supplement. For example, a tumor could be primed by direct injection of a chemical compound followed by later injection of the DNA, followed still later by an oral supplement of the same or different compounds.

In other embodiments, the invention provides methods for obtaining stabilized transient expression of foreign genes in a system of cell culture that perpetuates cells in semi-solid masses that simulate solid tumors, i.e., cells grown in a bioreactor. Protocols developed in this model tumor system can be used to transfect genes expressing anti-tumor compounds, e.g., IL-2, directly into solid tumors.

The mechanism by which the subject agents enhance transgenic expression has not been determined. They may directly stabilize the transfected DNA or its transcripts, or may even stabilize the expressed protein, though this latter possibility is unlikely. Moreover, Group I and Group II compounds appear to act through different mechanisms to enhance transient expression, since combinations of compounds from the two groups are often more effective than when the compounds are used separately (see, e.g., Example 6). In a preferred embodiment of the subject invention, the cells are contacted before, during, and after transfection with one or more compounds from

Group I, and are contacted following the transfection step with a compound of Group II, i.e., a sulfonated polysaccharide.

The following parameters have been defined to facilitate and characterize chemical compounds that are useful as agents for prolonging the duration of transient expression. These parameters are called the "*X*" factor, the "*G*" factor, and the "*K*" factor.

1. *X* factor: $X = 100 - \frac{(A \times 100))}{C}$,

where "A" is the amount of protein expressed in the control transfected cells during the chosen time period, and "C" is the amount of protein made in cells to which the chemical compound has been added.

This factor reflects the extent to which a chemical compound added to a transfected cell enhances stabilizes transient expression for the first four days following transfection. For chemical compounds active in stabilizing transient expression, the value for *X* will be >1. For example, if expression is doubled in the presence of a compound, $X = 50$. Preferred compounds will have $X > 10$, and most preferred compounds will have $X > 25$. This factor provides a way of comparing the amount of foreign gene expression observed when a chemical compound of the present invention is present in the culture medium for the first four days after transfection, as compared with the amount of expression observed in control cultures lacking the compound. Thus, the *X* factor is related to the ratio between the amount of expression observed in the presence and absence of the compound. *X* may be calculated similarly when the agent in question is a mixture of more than one chemical compound.

Cumulative protein expression, i.e., the values for "A" and "C", is measured by summing the values measured daily in aliquots of the cultured cells.

2. *G* factors. *G* factors differ from the *X* factor only with respect to the time period evaluated. For calculating a *G* factor, the amount of protein expressed is measured from days 4-7 or days 4-14, where day 0 is the day on which the foreign DNA is added to the cells. The subscripts denote which of the two time periods provided the basis for measurement. Thus, "G_7" indicates that the measurements were made between days 4-7, and "G_{14}" indicates that measurements were made between days 4-14. As for the *X* factor,

$$G_7 \text{ or } G_{14} = 100 - \frac{\text{A x 100}}{\text{C}},$$

where "A" and "C" are defined as for the *X* factor.

It is useful to characterize compounds according to both the *X* and *G* factors, because some compounds having low or negative values for *X* may have high or positive values for the *G* factors. Compounds with high values for G_7 or G_{14} are especially useful for transient expression where one or more compounds are added to the cultures after the DNA has already entered the cells, i.e., during the second phase of transient expression. Preferred compounds have values for $G > 0$. More preferably, $G > 10$, and most preferably, $G > 25$.

3. *K* factor. The *K* factor is the ratio of the rate constants for the decay of the foreign DNA expression in control transfected cells and in cells exposed to a chemical compound of the subject invention. *K* is determined according to the following equation:

$$K = \frac{k_{(DNA)control}}{k_{(DNA)compound}}$$

wherein "$k_{(DNA)}$" is the first order rate constant for the decay of expression of the transgenic DNA which is expressing protein as a function of time, i.e.,

$$\frac{-\text{d}_{(\text{DNA})}}{\text{dt}} = k_{(DNA)},$$

which is equivalent to $\log_{(\text{DNA})} = \frac{\text{kt}}{2.303} + \log_{(\text{DNA})}$. For convenience, the term "$\text{d}_{(\text{DNA})}$" is used as if it reflected changes in the effective concentration of transfected DNA, though it remains possible that the observed changes in expression are a function of some parameter other than simply the concentration of foreign DNA in the cells. Hence, the first order reaction rate in control transfected cultures can be expressed as $k_{(DNA)} = \text{d}_{(\text{DNA})}/\text{dt}$ = or $\log_{(\text{DNA})} = \text{kt}/2.303 + \log_{(\text{DNA})0}$. Thus, when $\log_{(\text{DNA})}$ is plotted against time, the intercept with the Y axis, or $\log_{(\text{DNA})0}$, reflects the initial concentration of transfected DNA that is expressed. Moreover, the slope of this line equals $-k_{(DNA)}/2.303$.

Values for $k_{(DNA)}$ are derived by using a computer program that plots the log of the foreign protein concentration against time. The program determines the slope of that portion of the resulting line that corresponds to the period during which

protein production is decreasing. Typically, the optimal amount of protein synthesis occurs during the 48 hour period following transfection. Thereafter, the rate of expression declines at a rate that is subject to manipulation by contacting the cells before, during, and/or after transfection with the various chemical compounds of the invention. Thus, the slope is calculated during this period of decline to provide values for $k_{(DNA)}$ and K that can be compared for the purpose of comparing the efficacy of different chemical compounds. For especially effective formulations of the subject chemical compounds, the initial decline in rate of expression may be followed by an increase in the rate.

The K factor thus reflects the effects of chemical compounds on the stability of the foreign gene expression after it is already inside the cell, and not the effects of these compounds on initial DNA uptake. The K factor is important because the advantages of this invention, in contrast with other reported methods for improving transient expression, derive primarily from providing a means for stabilizing transient expression after the transfection step, rather than on the traditional approach of trying to improve the efficiency of DNA uptake. However, some of that the chemical compounds of the subject invention have their maximal effectiveness during the first 4 days post-transfection, thus suggesting that they may act by inducing cells to take up increased amounts of the transfected DNA. Such compounds may act as well to prolong the effective half-life of gene expression once the foreign DNA is inside the cell. A positive value for K indicates that a compound or combination of compounds is effective in stabilizing foreign DNA expression post-transfection, thus the preferred chemical compounds and formulations of the invention will have values of $K>0$.

In the absence of the chemical compounds of the invention, the decay of transgenic DNA expression, i.e., $k_{(DNA)}$, is a first order reaction. When the chemical compounds of the invention are added to the cultures, the kinetics for expression of the foreign DNA change dramatically, as compared with control cultures. In the presence of these compounds, $k_{(DNA)}$ becomes increasingly more positive as the production of foreign protein is extended for long periods of time. Indeed, the changes are so dramatic for some of the most preferred formulations, i.e., those in which $K>40$, that conventional first order kinetics cannot adequately represent the results. Thus, it appears that the preferred compounds/formulations change the kinetics to either a pseudo-first order or a second order reaction. This result is not predicted by conventional wisdom.

Many compounds and formulations useful in the subject methods are discussed in the Examples and are included among those listed in these Tables 1, 8, 9, and 10, in which values for *X*, *G*, and *K* are presented.

This invention further provides a method based on the SW480 cell line for screening chemical agents to determine whether they are capable of stabilizing transient expression. For this method, candidate chemical compounds for screening are those that are biocompatible and that contain at least one hydrophobic moiety and at least one acidic moiety. The test compound or group of compounds is introduced into a culture of SW480 cells before, during, and/or after the introduction on day zero of foreign DNA that encodes a protein capable of being detected if it is expressed in the cells. For a period of days following the transfection step, samples of the culture are harvested and the amount of foreign protein therein is determined. The amount of the protein expressed cumulatively in the culture is determined by summing the amounts measured in the daily samples, and these sums are compared between test cultures, i.e., those that are contacted with the test compound, and parallel control cultures that are not contacted with the compound. Aliquots for protein measurement may be harvested daily between days 0 and 4, or between days 4 and 7, or between days 4 and 14, and the amounts of protein measured are used to determine, respectively, a value for X, G_7, or G_{14} according to the formulae given above. If the value thus determined for *X* or G_7 or G_{14} >0, it may be concluded that the agent is capable of enhancing transient expression. Preferably, *X*, G_7 or G_{14} >10, and most preferably, are >25. Chemical compounds so identified may be used to enhance the transient expression of foreign genes in the procedures described above. Other cell lines can be substituted for SW480 cells in this screening assay.

Preferred product formulations are selected from among those compounds and combinations of compounds that exhibit the highest (or most positive) values for the *X*, *G* and *K* factors. Furthermore, the various chemical compounds of the invention can be used together to maximize the enhancement of transgene expression. For example, the various compounds can be used to treat the same culture at different times during the procedure. Different formulations require different combinations of properties. Two distinctly different types of preferred formulations are:

Type A Formulations: These are compounds having a high value for *X*. These compounds are highly active immediately following the transfection step, and thus may act during the first phase of transient expression by enhancing the efficiency of DNA uptake. Therefore, the $\log_{(DNA)0}$, or Y intercept, from a semi-log plot as

described above, is higher in the case of a Type A compound or formulation than for the control culture without these compounds. Such compounds are assumed to affect the efficiency of DNA uptake because the Y intercept is a rough measure of the concentration of active foreign DNA inside the cell immediately following its introduction to the cell. Many of the compounds tested have positive values for the *X* factor (e.g., see Table 1). Thus, this invention not only provides chemical compounds for stabilizing transfected DNA, but also provides compounds that appear to enhance initial DNA uptake into the cell. Many of the treated compounds had high values for G_7 or G_{14} or *K as well as high values for X, thus are efficacious during* both phases of transient expression (e.g., see Tables 1, 3, 8, and 9).

Type B Formulations: Compounds useful in this category require both a high *G* and *K* factor. A high value for *X* is desirable, but is not required. Values for *K* that are greater than zero are characteristic of compounds capable of stabilizing transfected DNA. The most highly preferred Type B stabilizers have values of $K>1$, or more preferably, $K>10$, and values for G_7 or G_{14} >25. Furthermore, replicate experiments exhibiting *X* and/or *G* factors >25 are required before a particular agent is considered a highly preferred compound in either a Type A or Type B formulation.

For both *in vitro* and *in vivo* applications, transient expression is best maximized by the use of both Type A and Type B formulations. For example, a preferred method involves first priming the cells by exposing them prior to transfection to one or more Type A compounds that have a value of $X>25$. The Type A compounds are also present during transfection, and optimally remain present throughout the period of transient expression. After the transfection step, the cells are contacted for the remainder of the period of transient expression with one or more Type B compounds each of which preferably has a value for G_7 or $G_{14}>25$. In a preferred embodiment, the Type A compound is benzoate buffer, and the Type B compound is chondroitin-6-sulfate. In another preferred embodiment, the Type A compounds are benzoic acid and 4-ethylbenzoic acid, and the Type B compounds are benzoate buffer and chondroitin-6-sulfate. In yet another preferred embodiment, the Type A compounds are benzoate buffer and glutamic acid, and the Type B compound is chondroitin-6-sulfate.

For *in vitro* applications, the best results are achieved when cells are cultured in the presence of a Type A formulation for several hours, e.g., about 20-24 hours, prior to the transfection step. Note that priming with a Type A formulation in the form of a dietary (or oral application) is a realistic option *in vivo*. Many of the

compounds effective for enhancing transient infection are known to be non-toxic (see Table 1, below). Cultured cells are optimally maintained in the presence of the Type A formulation for at least 48 hours post-transfection. If desired, the Type A formulation can be removed after about >90% of the cells have taken up the foreign DNA, i.e., several days after the DNA is added to the culture, or this formulation can remain in contact with the cells during the second phase of transient expression. The Type B formulation is optimally added to the cells at the peak of transgene expression, which typically occurs 24-48 hours post-transfection. Optimally, feeding with medium containing a Type B formulation is repeated periodically for the duration of the experiment.

It should be apparent that the subject methods can be used *in vivo* (i.e., animal studies and clinical procedures). In particular, in the case of gene therapy involving a solid tumor, a Type A formulation may be co-administered with the DNA delivery vehicle, where the recipient tissue is "primed" by injection of a Type A formulation prior to administering the DNA. Thereafter, the Type B formulation is administered.

The useful concentration ranges for individual compounds may vary, and the upper limits of useful ranges may be limited by cytotoxic effects. Direct injection of the DNA/Type A formulation into a tumor would involve only routine procedures, as a variety of pharmaceutical carriers are well-known in the art. Direct injection would avoid exposing non-target tissues to the transfection reagents. Choline, liposomal formulations, or controlled release formulations can be combined with a Type B formulation to prolong the localized effect on the transfected tumor cells. In addition, a Type B formulation can be fed to the patient as a dietary supplement (or additive) for extended periods of time. Both injection and dietary feeding can be combined for optimal effectiveness according to factors such as toxicity, and the like. This approach offers the advantage of delivering high doses of a cytotoxic protein to a tumor without damaging other body tissues. Using this strategy, the tumor cells themselves would be induced to continuously produce the cytotoxic protein over a period of days, thus providing a far more effective means of delivery than simply injecting a dose of the protein itself into the tumor. This approach would be particularly helpful in cases where the protein in question is one that could not easily cross the plasma membrane if applied externally, or in cases where a therapeutic protein has a short half-life once inside the target cell.

In other manifestations of the invention, a compound selected from Group I may be linked covalently or non-covalently to a compound selected from Group II, e.g., chondroitin-6-sulfate.

Example 1.

Screening Assay for Enhancement of Transient Expression

Protocol for control transfections:

The following procedures were used to provide transient expression:

SW480 P3 (ATCC # CCL228) human colon carcinoma cells (typically, 1 x 10^6 cells) were plated in the wells of a 6-well tissue culture plate. The number of wells plated reflected the number of days post-transfection during which the experiment would proceed. Each well contained 1 ml of complete media from a 30 ml stock solution containing: 26.4 ml RPMI tissue culture medium, 4 mM L-glutamine, 3.0 ml fetal bovine serum, and 10 μg/ml gentamicin. Cells were cultured at 37°C in a CO_2 incubator with 10% CO_2 for 24 hours after being plated, during which time the cells adhered to the plates.

After the 24 hour pre-incubation step, the transfection step was carried out by removing the RPMI and adding 900 μL OPTI-MEM® (Gibco) medium containing 2 μg of VR1412 DNA, (Vical, Inc., San Diego, CA), which expresses the bacterial β-galactosidase gene under the control of a cytomegalovirus promoter, and 8 μg of a mixture of cationic lipid (1,2-dimyristyloxyproply-3-dimethyl-hydroxyethyl ammonium bromide (e.g., "DMRIE/DOPE") mixed in equimolar proportions with dioleoylphosphatidylethanolamine) to yield a lipid:DNA molar ratio of 0.99:1. It should be noted that typical transient transfection protocols employ 10 μg DNA per 10^6 cells, but the protocol described here uses less DNA in order to reduce toxicity to the cells. The plates were then incubated for 4 hours at 37°C.

After the 4 hour incubation step, 100 μl of heat deactivated fetal bovine serum (to stop transfection), plus 12.0 μl of 50 mg/ml gentamicin were added to each well. Twenty-four hours after the addition of foreign DNA to the wells, all of the cells from one well were trypsinized and counted, then 2 x 10^4 cells from each well were lysed and stored in liquid N_2 until being used at a later time to determine β-galactosidase concentration. At that time, each of the unharvested wells received 1 ml of the previously defined OPTI-MEM medium (without L-glutamine added). For the remainder of the experiment, one additional well was harvested at 24 hour intervals, and unharvested wells were fed 1 ml of OPTI-MEM (without L-glutamine, and containing the test compounds) every 48 hours.

Protocol for test compounds:

To test various compounds for their efficacy in enhancing transient expression, the protocol described above for control cultures was modified by incorporating the candidate chemical compound(s) into the culture media. The rest of the procedure remained unaltered with respect to the protocol for the control cultures.

Lysed samples from 2 x 10^4 cells were retained for each β-galactosidase assay, and the remaining cells from each well sacrificed daily. The lysates were frozen and maintained in liquid nitrogen until β-galactosidase assays could be conducted. The thawed samples were assayed for β-galactosidase using a chlorophenol red-based procedure based on chlorophenol red, wherein the colored product was quanitated at 580nm using an ultraviolet/visible light spectrophotometer.

The results from this assay for a large number of chemical compounds are presented below in Table 1. Table 1 gives values of X determined in experiments in which cultured SW480 human colon carcinoma cells were cultured and transfected with a bacterial β-galactosidase gene using the methods described in the Examples.

For purposes of comparison, Table 1 includes compounds that tested negative in the assay as well as a large number of compounds that tested positive. The pH values shown in Table 1 were determined in aqueous solutions made by diluting stock solutions prepared in culture media with deionized water. Compounds ranging from about pH 3 (melanin) to pH 10 (adrenaline) were observed to be effective for prolonging the duration of transient expression. The preferred pH range is about pH 4.5-9.0.

A test result was considered positive if the value calculated for any one of X, G, or K exceeded zero.

Table 1

GROUP I					
CHEMICAL COMPOUND	**mM**	**pH (H_20)**	***X* Factor**	***G_{14}* Factor**	***K* Factor**
'3-[BIS(2HYDROXYETHYL AMINO)]-2-HYDROXYL-'1-PROPANE SULFONIC ACID	1	6.81	-55	-10	
3-AMINO BENZENE SULFONIC ACID	1	3.8	-19	32	
3-METHYL-L-HISTIDINE	4	7.39	-15	55	
4-AMINO-BENZOIC ACID	1	7.67	52, 52	96*, -12	1, 1
4-BUTYLBENZOIC ACID	1	5.94	-26	65	9
4-ETHYLBENZOIC ACID	1	6	42, 46	43, 63	1, 2
4-HEXYLBENZOIC ACID	1	6.13	-68	-26	
4-OCTYL BENZOIC ACID	1	7.45	-2314	-665	
4-PENTYLBENZOIC ACID	1	1	-96	-88	
α-AMINO-n-BUTYRIC ACID	4	7.58	-.19	35	1
α-KETOGLUTARIC ACID	1	3.75	7.28	53	
ADRENALINE	1	10.29	49	68	
ASPARTIC ACID	4	5.75	40, -13	-45, -17*	
β-ALANINE	4	8.38	.2	33	1
α-ALANINE	4	7.27	31	35	
BENZOATE / HEPARIN	2.5, 0.1	5.51	-3	22	
BENZOATE BUFFER (equimolar benzoic acid/sodium benzoate)	4	4.74	29, 17, 3, 2, -16, 30	41*, 58, 27, 38, 44, 41*	2, 2, 1, 1, 1, 3*
BENZOIC ACID	1	4.21	-3.1	28	1

GROUP I					
CHEMICAL COMPOUND	**mM**	**pH (H_20)**	***X* Factor**	***G_{14}* Factor**	***K* Factor**
BENZOIC ACID & 4 ETHYLBENZOIC ACID	1, 1	5.82	41	80	2
BES	1	6.64	77,57	32,-24	1, 1
BUTYRATE BUFFER	2.5	6.12	-169, -275	81*, 69*	-9, 8
CARNOSINE	4	8.32	-8	35	
CITRULLINE	1	7.71	39	46	1
COENZYME B12	N/A	N/A	-17	51	
CREATINE	4	7.54	.34	28	
CYSTEINE	4	7.24	-35	-50	
DIIODOTYROSINE	4	7.23	-48	-8*	
ETHYL 4-ACETYLBENZOATE	1	7.59	49	42	1
ETHYL 4-ACETYLBUTYRATE	1	5.98	51	58	1
FOLIC ACID	1	6	-1.4	24	
GLUTAMIC ACID	1	4.2	-17, 8	-14*, 31*	2
GLUTAMIC ACID WITH BENZOATE BUFFER	1	4.91	44, 45, 3, 44, 42, 53, 44	69, 65, 27, 69, 76, 10, 69*	1, 1, 1, 2, 6*
GLUTARIC ACID	1	3.85	22	-77	
GLUTATHIONE	2	3.58	2	34	1
GLYCINE	4	7.27	-.34	40	2
HIPPURIC ACID	2	6.46	2	33	1
HISTIDINE	4	6.75	26, -30	54*, 6	1, 1
HOMOSERINE	1	7.4	77	39	
ISOLEUCINE	4	6.99	-119	30	

GROUP I					
CHEMICAL COMPOUND	**mM**	**pH (H_20)**	***X* Factor**	***G_{14}* Factor**	***K* Factor**
L-ARGININE	4	8.66	15, 54	11*, 57	
L-GLUTAMINE	4	7.14	-13	23	
L-THREONINE	4	8.14	28	40	1
LEUCINE	4	7.88	-13	32	
L-LYSINE	4	8.34	-39, 18	-10, 9*	
MELANIN	0.1	3.45	-155, 0	-453, -173	
METHYLCOBALAMIN	N/A	N/A	1	25	
METHIONINE	1	7.41	2	36	0
N-(4-AMINOBENZYL)-L-GLUTAMIC DIETHYLESTER	1	6.44	-35	8	
N-CARBAMYL-DL-ASPARTIC ACID	4	4.19	26	115	
N-FORMYL-L-METHIONINE	1	4.34	26	63	
NICOTINIC ACID	1	6.91	12	87	1
ORNITHINE	1	7.37	16	28	
PHENYLALANINE	4	6.97	-12	53	3
PROLINE	4	7.71	-22	21	
S-CARBAMYL-L-CYSTEINE	1	6.52	-76	-74	
SERINE	1	7.4	43	104	1
SODIUM BENZOATE	1	8.14	-15	-6	1
TAURINE	4	7.88	-20	34	2
TRYPTOPHAN	4	6.25	55	67	2
TYROSINE	4	7.88	36	52	2

GROUP I					
CHEMICAL COMPOUND	mM	pH (H_20)	*X* Factor	G_{14} Factor	*K* Factor
VALINE	4	8.12	-18	40	2

*Values for *G* denoted by an asterisk indicate values for G_7, while *G* values without an asterisk are values for G_{14}.

Note that those compounds in Table 1 for which $X > 1$ are compounds that increase the degree of transient expression during the first few days post-transfection. Such compounds may influence cells to take up larger amounts of DNA per cell than they otherwise would, or alternatively, may cause a higher proportion of transfected cells to express the foreign DNA than otherwise would have expressed it. It remains possible also that these compounds enhance early transcription or expression. These compounds have not been previously reported to have this effect on transfection. Interestingly, melanin was noted to significantly suppress transient expression.

In addition to those compounds listed in Table 1, additional compounds that have been tested and found capable of prolonging transient expression include t-butyl benzoic acid, ethyoxy benzoic acid, iso-propyl benzoic acid, methoxy benzoic acid, isobutyl benzoic acid, chondroitin-6-sulfate (type C), and guarans, particularly hydroxypropyl guaran.

Example 2.

Chemical Compounds Enhance Transient Expression and Reduce Glucose Consumption

Additional experiments were performed to further characterize the enhanced transient expression method. For these experiments, the five culture conditions described in Table 2 were tested using the transient expression protocol described in Example 1. Six-well plates were used, and a sufficient number of wells were seeded with SW480 cells so that the cells from individual wells could be harvested as described below.

Table 2

Plate #	Chemical Compounds	Concentration	Media Type
1A	Control with gentamicin	--	see above
1B	Control with no gentamicin	see above	see above
2	Benzoate buffer L-glutamine	2.5 mM 4 mM	Pre-transfection Transfection Post-transfection feeding
3	Chondroitin sulfate (type C) Benzoate buffer L-glutamine	0.1 mM 2.5 mM 4 mM	Pre-transfection Transfection Post-transfection Feeding
4	Glutamic acid Benzoate buffer L-glutamine	4 mM 2.5 mM 4 mM	Pre-transfection Transfection Post-transfection Feeding

Each day, one well was harvested for counting, and 2 x 10^4 cells from each harvested well were lysed, and the lysates retained for a β-galactosidase assay. Supernatants from these same wells were retained frozen and used later for evaluation of pH, glucose consumption, and the production of lactate and ammonia. As seen in Table 3 below, the various combinations of chemical compounds used in plates 2, 3, and 4 differed in their ability to enhance and sustain gene expression. Plate 4 had the best overall performance in this experiment, with high *X* and *G* factors. Plate 3, the only plate in this experiment that included a Group II compound, clearly showed signs of reduced transfection efficiency (i.e., a low *X* factor) but showed promise for sustained expression (i.e., a relatively high *G* factor).

Table 3

	Plate Number				
	1A	1B	2	3	4
Parameter	gentamicin	no gentamicin			
X factor	n/a	9	30	-26	44
G factor	n/a	32	41	51	69
Time elapsed	**% X-gal (blue)**				
24 hr	60-70	85-90	80-90	85-95	95-100
48 hr	40-50	60	50-60	70	80
72 hr	40-50	60	60-70	60	70-75
96 hr	10-20	30	50-60	55-60	30
120 hr	10-20	20-30	30-40	20-30	30
144 hr	10-15	10-20	20	25	20-30
168 hr	10-15	2-5	10	10	2-5

Cell culture experiments typically show a standard deviation in the range of 20%. For this reason, *X* and *G* factors less than 25 were not considered to be significant improvements over the control.

A control experiment (plates 1A and 1B, Table 3) was included in the above-described experimental scheme to determine whether gentamicin, an antibiotic present in the culture media, may have influenced the outcome of the experiments described above. From comparing the results for control plates #s 1A and 1B, it is evident that gentamicin somewhat suppressed protein production. This is suggested by the slightly lower values for *X* and *G* factors in controls with gentamicin, i.e., plate 1A, as compared with plate 1B, the control without gentamicin. Furthermore, results from the β-galactosidase assays supported this conclusion.

Glucose consumption and lactate production, as well as ammonia production, in these same cell samples were analyzed. Glucose and lactate were measured using a Kodak Ektachem DT60 II Analyzer according to standard protocols provided by

Kodak and routinely used for measuring serum glucose and lactate levels in clinical laboratories. The analyses are conducted by applying 10 µl of each test sample to a well on a plastic slide covered with a film containing all the reagents necessary for measuring either glucose or lactate (Ektachem DT slide (GLU) or Ektachem DT Slide (LAC)). For measuring glucose, the analysis is based on the glucose oxidase-catalyzed reaction of glucose with molecular oxygen, followed by a second reaction that produces a red dye whose intensity is proportional to the amount of glucose in the sample. The slide for measuring lactate similarly provides enzymes and substrates capable of producing a red dye in an amount proportional to the amount of lactate applied to the slide. Slides are placed in the Ektachem DT60 II Analyzer in which the red color is read by reflectance spectrophotometry. Ammonia analysis was performed similarly, using Ektachem DT slides (NH_3), based on a reaction wherein NH_3 reacts with bromphenol blue to yield a blue dye detectable with the same instrument.

The results of measuring glucose and lactate concentrations as a function of time are presented in Table 4. Table 4 indicates, surprisingly, that the control with gentamicin (plate 1A) consumed more glucose and produced more lactate than any of the experimental samples, which also contained gentamicin (note that the control without gentamicin, i.e., plate 1B, is not included in Table 4). The data of Table 4 provide a clear indication that relative to the control the cells that received the chemical compounds described in Table 2 experienced a profound shift in metabolism that corresponded with a substantially higher level of expression of the foreign gene.

In addition to the data in Table 4, a combination of benzoic acid and 4-ethylbenzoic acid also have been observed to result in reduced glucose consumption. Here, an experiment was conducted in which a Type A formulation was first applied to SW480 cells before and during the transfection step, and a Type B formulation added one day after the DNA was introduced into the cells. The Type A formulation consisted of OPTI-MEM containing 1 mM benzoic acid, 1 mM 4-ethylbenzoate, and 4 mM L-glutamine, while the Type B formulation contained these same components and in addition contained 0.1 mM type C chondroitin-6-sulfate. Gentomicin was also present throughout the experiment. In this experiment, essentially no glucose consumption was observed in cells cultured in 6-well plates for as long as 14 days post-transfection, or for as long as 32 days post-transfection in bioreactors, during which time the cells continued to express protein from the transfected DNA.

Table 4

Plate #	Day	Glucose Concentration (mg/dL)	Lactate Concentration (mmol/L)
1A.	0	218	1.5
	2	180	6.0
	4	141	9.6
	6	38	>12.0
2.	0	209	1.8
	2	188	5.0
	4	175	6.5
	6	---	---
3.	0	213	1.6
	2	195	4.3
	4	185	6.1
	6	---	---
4.	0	209	1.6
	2	199	3.8
	4	181	5.7
	6	140	9.5

It has been previously reported that butyrate, a Group I compound, when administered to cultured hepatocytes compensates for the effects of glucose-starvation on post-translational glycosylation, most likely increasing the intracellular glucose pool (Morrow et al., *Biochem. Biophys. Res. Comm.* 112:115-125 (1983)). However, Morrow et al. did not assay the consumption of glucose in their cultures, thus did not observe the shift in metabolism that is noted here in the presence of Group I compounds. The observed shift in glucose metabolism is a highly significant feature of this invention. Not only does it correlate with the enhanced efficacy of

chemical compounds relevant to gene therapy methods (as is evident from this example), but suggests that the ability to selectively and non-toxically redirect cellular metabolic processes with these same chemical compounds could be applied to a wide range of therapies, including, for example, the modulation of fat/lipid metabolism in treating obesity.

Example 3.

Enhanced Transient Expression in Bioreactors

A series of four lipofection-based gene transfection experiments were conducted in a high performance hollow fiber perfusion prototype bioreactor device (hereafter referred to as the "HPBr" device) in a Genespan prototype incubator instrument. The device consists essentially of a sterile chamber through which two sets of hollow fibers are passed. Culture medium is continuously circulated through one set of fibers, while gases required (e.g., oxygen and carbon dioxide) for cell growth are passed through the second set of fibers. The fibers are composed of a porous material through which gases and nutrients can pass in one direction, while waste molecules produced by the cells growing within the chamber can pass in the other direction. Cells growing in the device may remain in suspension, or may attach to the outer surfaces of both sets of hollow fibers.

A useful feature of the HPBr device is that the cells can be agitated by rotating the chamber through which the tubes pass. When the chamber is rotated 120° in one direction around its longitudinal axis, then 120° in the other direction, this constitutes one "cycle" of rotation. Alternatively, cultures can be grown under "static" conditions, using no rotation.

The HPBr device was used to conduct a series of experiments using SW480 cells. Each experiment included a parallel control in which the cells were plated in a conventional 6-well plate that was placed in a conventional 10% CO_2 incubator. The control 6-well plates were cultured and transfected using the protocol described above for the control plates in Example 1, while the following experimental procedures were employed for the bioreactor devices.

HPBr Device Experiments

Four β-galactosidase reporter gene transfection experiments were conducted in HPBr devices using a protocol similar to that described in Example 1 for 6-well plates, although volumes of the various reagents had to be adjusted proportionately to accommodate the larger volumes and higher number of cells in the bioreactors. Due

to the perfusion mode of cell culture which is characteristic of the HPBr (i.e., continuous feeding), there was no requirement for periodic feeding by hand.

Procedures for the bioreactor experiments differed in the following ways from the procedures described in Example 1. Sufficient Cytodex® 1 microcarriers (i.e., microspheres composed of crosslinked dextran with positively charged quaternary ammonium functional groups on the surface for cell attachment; Sigma, St. Louis, MO) were pre-swollen in phosphate-buffered saline and introduced into the side ports of the HPBr. Approximately 1 microcarrier bead per 10 cells were used. At the onset of the experiment, 1×10^7 viable SW480 cells and 1×10^6 beads were co-injected into the device. The media described in Table 5 were present when the cells were seeded into the device. Table 5 identifies the rotational parameter ("cpm," corresponding to cycles per minute) employed in this study. A volume of 839 ml of medium were added to each bioreactor. Type C chondroitin sulfate at 0.1 mM was included in the OPTI-MEM transfection media for runs 2, 3, and 4 ("runs" refer to separate experiments). Following the transfection step, the recirculating OPTI-MEM medium (i.e., the medium inside the tubes) was replaced, but the medium in the compartment containing the cells (the extracapillary space) was not replaced. The replacement medium included the compounds listed in Table 5. Liposomes containing the foreign DNA were added to the extracapillary space 24 hours after the cells were seeded into the bioreactors. This space has a small volume (17 ml) as compared with the volume inside the tubes (839 ml).

Table 5

RUN	TYPE	CONDITION	MEDIA COMPOSITION
1.	Plate	CO_2 Incubator [control]	OPTI-MEM (see Example 1)
2.	HPBr	30 cpm	OPTI-MEM; 10% fetal bovine serum; 4 mM L-glutamine; 10 g/ml gentamicin; 2.5 mM benzoate buffer; 0.1 mM chondroitin sulfate (type C) [also present in OPTI-MEM transfection media].
3.	HPBr	Static	Same as run #2.
4.	HPBr	30 cpm for first 48 hr., then static	Same as run #2.
5.	HPBr	Static [control]	OPTI-MEM

Daily samples (about 1.5 ml) of cells and supernatant were taken from the cell compartment of each bioreactor and an equal volume of fresh media was added to replace it. Cell counts and viabilities were determined, and 2 x 10^4 viable cells were lysed and retained for β-galactosidase determination using the spectrophotometric method described in Example 1.

Table 6 contains data comparing the results from four perfusion device experiments (runs #2-5) with a plate control (run #1). In Table 6, the column labeled "area under the curve" refers to the area under a curve in which the amount of β-galactosidase produced in the daily aliquots of harvested cells were plotted as a function of time for the two-week duration of this experiment. Thus, the values in the "area under the curve" column thus are expressed in arbitrary units, i.e., cm^2, and reflect the total amount of β-galactosidase produced on a per cell basis for the duration of the experiment. The last column in Table 6 shows for each run, i.e., each plate or bioreactor, the sum amount of β-galactosidase present at day 13 in all of the viable cells remaining at that time.

It is evident that the perfusion bioreactor can be employed to scale-up gene transfection and harvesting transfected cells, which is advantageous for therapeutic applications (e.g., for creating large numbers of T-lymphocytes and hematopoietic

stem cells expressing foreign genes either stably or transiently, e.g., to be used in somatic cell therapy). This system can also be utilized as an artificial organ so that the long-term expression of the foreign gene can be easily and realistically studied; in a way, this is equivalent to taking a biopsy from an intact organ *in vivo*.

Table 6:

2-Week β-Galactosidase Production in Plate and HPBr Device

Run	Experimental Conditions	Total # Cells (13 Days)	% Viability	Area Under Curve (cm^2)	β-Galactosidase Expression per $2x10^4$ Cells as % of Control	ng/ml β-Gal per $2x10^4$ Cells at Day 13	Total Expression Based on Viable Cells at Day 13
1	Plate Control	6.5×10^6	97%	70	-	0.084	33
2	30 cpm	1×10^7	86%	84	20%	0.120	65
3	Static	2.3×10^7	42%	105	50%	0.602	364
4	30 cpm/48 hr then static	7.3×10^7	77%	180	157%	0.357	1254
5	Bioreactor Control (Static)	29.3×10^7	34%	76	9%	0.040	249

The data in Table 6 show that manipulating the rotational parameter of the bioreactor provides a unique and convenient means for enhancing transfection efficiency and sustained transient expression in using this device.

It is notable that the presence of type C chondroitin sulfate, a polyanionic carbohydrate, allowed the transfection to proceed unimpeded, and it also resulted in a substantial improvement in gene expression. Some of the other polyanionic carbohydrates tested actively blocked the transfection process. These included Type A chondroitin sulfate, dermatan sulfate, heparin sulfate, heparin, carboxymethylcellulose, and N-carboxymethylchitosan N,S-sulfate (Table 1). Therefore, the tolerance of cells to type C chondroitin sulfate is not typical of their tolerance in general for polysaccharides in the culture medium.

As discussed above, microspheres can be introduced into the chamber in order to provide attachment sites for cells. It has been observed, for example, that when an immortal mouse melanoma cell (i.e., ATCC #B16-F0) is introduced into the chamber with microspheres present, the microspheres act as "seeds" for the accumulation of large masses of cells. It was further observed that these masses of cells could be transfected and that thereafter the cells in these masses transiently express the transfected DNA. Samples are readily obtainable from such cultures by sampling the medium within the chamber. This sampling is accomplished by directing a flow of fresh media from a syringe against the cell mass, which results in a number of cells sufficient for sampling becoming suspended in the medium. The masses of cells resemble a solid tumor and provide a model system for developing therapeutic methods effective in delivering therapeutic proteins to tumors *in vivo*.

Using the same protocols that are effective for cell masses growing inside the bioreactor, melanoma cells will be injected subcutaneously into mice, allowed to develop into tumors at the site of injection, and then liposomes containing the β-galactosidase vector DNA will be introduced directly into the tumors to achieve transient expression of the β-galactosidase. The methods effective for expression of β-galactosidase are expected to be effective for other proteins as well, and similar experiments will be conduced to evaluate the effects of delivering various proteins, e.g., DNA encoding therapeutic proteins, directly into solid cell masses *in vivo*.

The bioreactor system used with the methods of the subject invention is useful for creating large numbers of cells genetically modified to express a foreign protein. Such cells can be administered to patients for therapeutic purposes and maintained thereafter in an active state only for as long as the therapeutic regimen dictates. Thus,

the subject invention provides a unique form of gene therapy wherein the introduced gene can be turned off simply by restricting its access to the stabilizing substances, i.e., by administering cells transiently expressing a therapeutic protein, then administering the enhancing compounds for only so long as continued transgene expression is desired.

Finally, it should be noted that the use of chondroitin-sulfate (type C) is important as it enabled the anchorage-dependent cells to adhere well to the microcarrier despite the relatively high rotational speed. This results indicates that compounds of Group II are useful for providing anchorage to solid substrata for cultured cells. Chondroitin sulfate has been proposed as a compound for providing a cell adhesive surface in a device for controlling the pattern of cells on a surface (U.S. 5,593,814). However, the method of U.S. 5,593,814, in contrast to the subject procedure, requires that the chondroitin sulfate be bonded to the solid substratum, rather than being added to the culture medium. Others have reported using chondroitin sulfate in conjunction with other compounds in order to promote cell adhesion in culture or *in vivo*. (U.S. 5,593,814; U.S. 4,458,678; U.S. 4,418,691; U.S. 4,711,780; U.S. 5,545,722).

Example 4.

Assay for Cytotoxicity

A number of chemical compounds were tested in 6-well plates according to the protocol described in Example 1 to determine the relationship between their cytotoxicity and their ability to promote the uptake and expression of foreign genes in SW480 cells. Unless otherwise noted, except for the control, all plates contained 4 mM L-glutamine as well as gentamicin to retard bacterial growth.

Cytotoxicity assays were performed as follows. SW480 cells (approximately 1×10^6 cells per well) were plated in 1 ml of RPMI in 6-well culture dishes at day zero in the presence of the chemical compound whose cytotoxicity was being tested. Twenty-four hours after seeding the wells, the RPMI medium was removed, and liposomes containing foreign DNA were added to the culture in 1 ml of OPTI-MEM medium, as described in Example 1. The transfection medium also contained the chemical compounds whose cytotoxicity was being tested. Control plates were included that were identical to the test plates, except that the test compounds were not present in the culture medium. Test and control cultures were grown under "static" conditions, i.e., the plates were not shaken, rotated, or otherwise agitated. Each day for a total of 8 days, the cells from one test well and one control well were

harvested and viability assessed by staining with trypan blue. In control cultures exposed to liposomal DNA, the cell number remained fairly constant or increased only slightly for the first 4 days post-transfection, then increased to about 1 x 10^7 per well by the end of 8 days. The retardation of growth of control cultures during the first 4 days was presumably due to the mild cytotoxic effects of the liposomal DNA itself. A compound being tested was considered "cytotoxic" at the test concentration if a >50% decline in the number of viable cells was observed within 4 days after the introduction of foreign DNA, and furthermore, there was no net expansion of the cells at the end of 8 days.

By applying this test protocol, it was possible in many cases to manipulate the concentrations of individual compounds or formulations of compounds to arrive at concentrations well-tolerated by SW480 cells yet also capable of enhancing the levels of transient expression in these cells. Other cell types also were tested for their ability to tolerate some of the chemical compounds of the subject invention. For example, human melanoma cells, mouse melanoma cells, and COS-7 cells (ATCC CRL 1651) were tested for their ability to tolerate the formulations applied to plate #6 in Table 9. The cells differed somewhat in their sensitivity to the tested compounds, but a set of concentrations was identified that could be tolerated by all of these cell types, i.e., at these concentrations the compounds were not cytotoxic according to the above-described assay.

Sulfonated amino polysaccharides that enhanced transient expression were all found capable of supporting normal cell growth, i.e., they were not too toxic to be tolerated by the cells. The cell growth and cytotoxicity curves for the cells exposed to the various chemical compounds and formulations in Table 7 are presented in FIGURES 1 and 2, in which the numbers describing each plot correspond to the plate numbers in Table 7. Table 7 illustrates that the polysaccharide heparin blocks transient expression, but that type C chondroitin-6-sulfate did enhance transient expression. Although not shown in Table 7, it has also been observed that guarans also enhance transient expression. The heparin-mediated suppression of gene expression may have resulted from the formation of complexes between heparin and the cationic lipid in the liposomes, thus leaving the DNA without a carrier to deliver it to the cells. In light of this inference, the ability of chondroitin-6-sulfate to support both gene expression and cell growth is surprising.

Table 7

Plate #	Compound/Formulation	Group	Transgene Expression	Cytotoxic
1.	Control 4mM L-glutamine	n/a	Yes	No
2.	2.5 mM benzoate buffer ~0.1 mM chondroitin-6-sulfate (type C) 1mM L-glutamine 4.0mM L-glutamine	I & II	Yes	No
3.	2.5 mM benzoate buffer ~0.1 mM heparin 1mM L-glutamine 4mM L-glutamine	I & II	No	No
4.	~0.1 mM heparin 1mM L-glutamine 4mM L-glutamine	II	No	No
5.	0.1 mM chondroitin-6-sulfate (type C) 1mM L-glutamine 4mM L-glutamine	II	Yes	No
6.	2.5 mM butyrate buffer	I	Yes	Yes
7.	2.5 mM butyrate buffer 1mM L-glutamine 4mM L-glutamine	I	Yes	Yes

The plates containing butyrate buffer expressed the transfected gene, however, this buffer was cytotoxic to the SW480 cells under the experimental conditions used for this set of experiments.

Example 5.

Transfection with Starburst Polymers

This set of experiments addressed the issue of whether the efficacy of the subject methods for enhancing transient expression were dependent on the means by which the DNA is delivered to the cells. Two different combinations of chemical

compounds (see Table 8) were employed in transfecting SW480 cells using a protocol similar to that in Example 1, except that here the DNA was introduced into the cells in the presence of polymeric dendrimers instead of using liposomal delivery. These dendrimers are microscopic synthetic polymer spheres (first commercialized by Dow Chemicals as "starburst" polymeric bead standards to be used for sizing), which can be chemically derivatized to play the role of a cationic lipid. The dendrimers employed in this example were provided by F.C. Szoka, Jr., Department of Pharmacy/Pharmaceutical Chemistry, University of California, San Francisco, CA. While the detailed mechanism of gene delivery for either lipofection or dendrimer-mediated processes is not known, based on physicochemical properties such as their shape and distribution of chemical moieties, they are highly likely to be quite different.

The procedure used deviated from that of Example 1 in the following steps. Fourteen μg DNA were diluted into 397 μl deionized water, and 56 μg of the dendrimer was diluted into 393 μl deionized water. The DNA solution and dendrimer suspension were combined no more than one hour before use. OPTI-MEM medium (733 μl) and the DNA/dendrimer mix (167μl) were added to each well, and the 6-well plates were swirled by hand to ensure thorough mixing. After incubating for 5 hours, the DNA/dendrimer-containing media was removed and 1.0 ml of culture medium was added. The remaining steps in the procedure were as described in Example 1.

As illustrated in Table 8, the tested compounds were efficacious when dendrimers were used as the method for delivering the foreign DNA to the cells. These findings strongly suggest that the formulation of chemical compounds shown in Table 8 exert their effect after the DNA enters the cell, and thus are effective regardless of the method used to introduce the DNA.

Table 8

Plate #	Compound/Formulation	*X* Factor	*G* Factor	*K* Factor
1.	Control	n/a	n/a	n/a
2.	2.5 mM benzoate buffer 0.1 mM chondroitin-6-sulfate (type C) 4 mM L-glutamine	14	63	2
3.	2.5 mM benzoate buffer 4 mM glutamic acid 4 mM L-glutamine	42	34	-2

Example 6.

Protein Production During Transient Expression

The following experiment illustrates that the subject transient expression system is useful for the rapid production of large amounts of a protein product expressed by a foreign gene that is introduced into recipient cells using the methods described in the preceding examples.

A 15 plate experiment was conducted in which the chemical compounds indicated in Table 9 were added to the culture medium of SW480 cells that were transfected in 6-well plates as detailed in Example 1. The *X*, G_{14} and *K* factors, plus the cumulative amount of protein produced in 14 days in 2 x 10^4 cells, were calculated and are shown in the last column of Table 9. The data presented in Table 9 illustrate that all of the listed compositions were superior to the control with respect to the amount of protein produced in their presence. The most efficacious formulations, in order of their effectiveness, were those used in plates 6, 3, 13 and 14. Superior results were observed in the plates that received both Type A and B formulations, thus, these combinations are especially useful for animal testing, e.g., as in treating tumors with toxic proteins, delivering hormones to specific tissues, or other pathological conditions where local delivery of a bioactive protein may be desirable.

Table 9

Plate #	Compound/Formulation	*X* Factor	G_{14} Factor	*K* Factor	Total Protein (ng per 2 x 10^4 cells)
1.	control (DNA but no compounds)	n/a	n/a	n/a	10.2
2.	2.5 mM benzoate buffer	-16	43	1	11.7
3.	2.5 mM benzoate buffer *Cells fed after 48 hr with Type B Formulation:* 2.5 mM benzoate buffer 0.1 mM chondroitin-6-sulfate	-14	63	2	28.7
4.	4 mM tryptophan	55	67	2	34.2
5.	1 mM benzoic acid 1 mM 4-ethylbenzoic acid	41	80	2	27.5
6.	*Type A Formulation* 1 mM benzoic acid 1 mM 4-ethylbenzoic acid *Cells fed after 48 hr with Type B Formulation:* 2.5 mM benzoate buffer 0.1 mM chondroitin-6-sulfate	20	82	42	26.2
7.	1 mM 4-ethylbenzoic acid	47	64	2	21.9
8.	1 mM 4-butylbenzoic acid	-26	65	9	14.6
9.	4 mM L-glutamine	-12	42	---	11.7
10.	4 mM citrulline	40	46	1	17.5
11.	4 mM benzoate buffer 0.1 mM chondroitin-6-sulfate	54	72	2	26.1

Plate #	Compound/Formulation	*X* Factor	G_{14} Factor	*K* Factor	Total Protein (ng per 2 x 10^4 cells)
12.	2.5 mM benzoate buffer 4 mM glutamic acid	42	76	6	25.1
13.	*Type A Formulation* 2.5 mM benzoate buffer 4 mM glutamic acid *Cells fed after 48 hr with Type B Formulation:* 2.5 mM benzoate buffer 0.1 mM chondroitin-6-sulfate	49	78	---	28.2
14.	*Type A Formulation*: 1 mM glutathione 1 mM methionine 4 mM glycine 4 mM α-amino-n-butyric acid 1 mM taurine 4 mM phenylalanine 2.5 mM benzoate buffer 4 mM alanine	57	77	1	29.6
15.	1 mM ethyl-4-acetylbutyrate	51	59	1	22.0

Note: Medium in all plates included gentamicin, and except for the control, also contained 4 mM L-glutamine; the chondroitin-6-sulfate was type C.

These experiments illustrate also the utility of enhanced transient expression for very rapidly producing milligram quantities of protein without the need to first establish cell lines into which the foreign gene has become stably integrated. Thus, enhanced transient expression provides a new means by which candidate biopharmaceuticals can be efficaciously expressed in sufficient quantities to be recovered and rapidly screened for pharmaceutical activity. Thus, the subject invention provides a means for implementing an accelerated drug discovery program. Plate 6, for example, produced about 26 ng β-galactosidase per 2 x 10^4 cells in 14 days (see Table 9). Scaled up to a conventional culture containing around 2 x

10^6 cells, the cumulative protein production using this formulation would be about 26 mg. In the HPBr device employed in Example 2, as many as 10^9 cells are routinely grown, thus in such a culture, tens or even hundreds of milligrams of a novel or interesting protein could be obtained within a matter of a few days.

Example 7.

Transient Expression in Hepatocytes

A totipotent (stem-cell like) clonal nontransformed cell line (PICM-19 3BT cells; hereafter referred to as "PICM-19 cells") derived from pig embryonic cells (epiblast stage), was obtained from Dr. N. Talbots (U.S.D.A., Beltsville, MA). These cells behave like hepatic stem cells, showing self-renewing properties for many months when cultured in the presence of 5% or less CO_2. At higher levels of CO_2, (e.g., up to about 10%), these cells begin to differentiate. At least two different differentiated cell phenotypes have been isolated from differentiated PICM-19 cells, namely, mature hepatocytes and liver ductile cells, which produce bile. PICM-19 cells that had been induced to differentiate were used as a means for determining the transfection characteristics of primary hepatocytes, a cell type that they strongly resemble. In earlier experiments with primary pig liver cultures, results were obtained that mirrored those described above for the SW480 cells. Because the primary liver cultures contained cell types other than hepatocytes, the experiments were repeated with PICM-19 cells providing a homogeneous source of hepatocyte-like cells.

The protocol employed was identical to that described in Example 1 used for transfecting SW480 cells, using 1×10^7 cells per well, except that the PICM-19 cells were plated on a layer of mytomicin C-inactivated STO mouse fibroblast feeder cells (CRL 1503), without which PICM-19 cells normally will not grow. In preparing liposomes, the DNA/lipid to cell ratio was as in Example 1. The incubator was maintained at 10% CO_2 throughout these experiments. The PICM-19 cells expanded and under these culture conditions differentiated into mature hepatocytes. To ensure that the differentiation was complete, the cultures were maintained in 10% CO_2 for 3 weeks prior to the transfection step.

Table 10 describes the media that were used in a transfection study using these cells, as well as the X, G_7 and K factors measured in these cultures. The results shown in Table 10 are consistent with the findings for SW480 cells and the results observed when primary isolates from adult pig liver were transfected under similar conditions.

Surprisingly, it was observed also that the plates lacking feeder cells were capable of supporting differentiated PICM-19 cells for at least 4 weeks. These cells moreover expressed the transfected DNA, as illustrated in FIGURE 1. This result was extremely surprising, as there are no reports of hepatocytes being grown or maintained in culture for more than a few days without either a feeder layer or a proteinaceous coating (e.g., collagen) having been applied to the plates prior to adding the cells. Remarkably, the cells in plate #4 adhered as well as did cells in plates containing feeder cells, suggesting that the type C chondroitin-6-sulfate created in vivo-like conditions for both cell growth and maintenance. Thus, these experiments demonstrate for the first time the utility of chondroitin sulfate for culturing hepatocytes without feeder cells in a low cost medium composition while maintaining a phenotype similar to that observed for hepatocytes in vivo.

Table 10

Plate #	Compound/Formulation	*X* Factor	G_7 Factor	*K* Factor
1.	Control	n/a	n/a	n/a
2.	2.5 mM benzoic acid 4 mM L-glutamine	-17	99	1
3.	2.5 mM benzoate buffer 0.1 mM chondroitin-6-sulfate (type C) 4 mM L-glutamine	-41	102	0.0
4.	2.5 mM benzoate buffer 0.1 mM chondroitin-6-sulfate (type C) 4 mM L-glutamine ***NO FEEDER CELLS***	-357	103	2
5.	2.5 mM benzoate buffer 4 mM glutamic acid 4 mM L-glutamine	-46	103	0.0

Example 8.

Recovery of Transgenic mRNA and DNA from Transfected Cells Grown in a Bioreactor

The high performance bioreactor device (HPBr) described in Example 3 was used in a 32-day experiment in which SW480 cells were transfected and propagated as described in Example 3 and in Table 5. Except as described otherwise below, the conditions and assays used were the same as described in Example 3. At the onset of the experiment, 1 x 10^7 SW480 cells freshly harvested from tissue culture flasks were injected into the HPBr device concurrently with 1 x 10^6 preswollen microspheres. The cells were then cultured for 24 hours without rotation in medium containing 1 mM benzoic acid and 1 mM 4-ethylbenzoic acid (a Type A formulation). At the end of 24 hours, plasmid DNA encoding β-galactosidase was added, and the bioreactor was rotated at a rate of 30 cpm for 4 hours. The medium containing the DNA was then removed from the extra-capillary space (ECS) of the bioreactor by flushing three times with feeding medium containing 1 mM benzoic acid, 1 mM 4-ethlybenzoic acid,

and 0.1 mM chondroitin-6-sulfate (a Type B formulation). Thereafter, the 1 liter bottle of culture medium for circulating through the bioreactor was replaced with a 1 liter bottle of feeding medium containing the same Type B formulation. For the remainder of the experiment, the medium circulating through the bioreactor was replaced every 7 days with a fresh 1 liter bottle of feeding medium containing the Type B formulation. The device was not rotated after the DNA was removed so that the cells could form a tumor-like solid mass.

Beginning 24 hours after removing the DNA from the bioreactor, aliquots of cells and culture supernatant from the ECS were removed daily for 32 days. Cell sampling was accomplished by directing a stream of culture medium against the cell mass to dislodge some of the cells, then withdrawing a small volume of the resulting cell suspension. The cells and culture medium in each sample were separated by brief centrifugation. A total of 2×10^4 cells from each daily aliquot were analyzed for β-galactosidase and each supernatant was analyzed for its metabolic signature, i.e., its concentrations of glucose, lactate, and ammonia. After collecting the daily sample on day 32, the remaining cells were harvested from the ECS by trypsinization, and 2.8×10^5 of the harvested cells were used for the extraction of RNA and DNA.

Beta-galactosidase in the daily cell samples was assayed as described in Example 3, and the results of these assays are illustrated in FIGURE 4. FIGURE 4 shows that the peak level of expression of β-galactosidase occurred at day 4, and remained virtually unchanged until about day 12, whereafter the values became less consistent but nonetheless remained relatively high. The final data point, corresponding to cells collected by trypsinization at the end of the experiment, is indicated in FIGURE 4 by a square-shaped symbol, and its value corresponded to roughly 60% of the peak value. Thus, a relatively high level of β-galactosidase production took place in this culture throughout the entire 32-day period.

The procedures described in Example 2 were used to measure the concentrations of glucose, lactate, and ammonia in the supernatants, and the results of these measurements are presented in FIGURES 5A-5C. It is apparent from FIGURES 5A and 5B that neither the glucose nor the lactate concentrations changed to a significant extent throughout the course of the experiment (the fluctuations in lactate were not considered significant in view of the low amounts of lactate present in these samples and in view of the relatively constant amounts measured past day 7). In contrast, the ammonia concentration increased over two-fold by the end of each seven-day period between media changes, before dropping back to the base value

each time fresh medium was provided. This repeated accumulation of ammonia after each medium change strongly supports the notion that exposure to transfection-stabilizing compounds causes cells to shift their metabolism from using glucose (glycolysis) to using proteins or amino acids instead as their primary carbon source (tricarboxylic acid cycle). Had the cells in this experiment used glucose for their primary source of energy, one would have expected lactate and not ammonia to increase in concentration during each 7-day period (note that FIGURE 5B suggests that some glycolysis may have occurred during the first 7-day period).

Ammonia is a byproduct of the deamination that is an early step in the entry of amino acid metabolites into the tricarboxylic acid cycle. Accordingly, the most likely explanation for the accumulation of ammonia in the culture media is that the cells used amino acids, or possibly peptides or proteins, as their source of energy during their exposure to the compounds used to stabilize transient expression. These amino acids may have originated, for example, from peptides present in the culture medium. Such peptides could have been created by the heat-induced breakdown of serum proteins during the heat inactivation of the serum present in the culture medium.

Their ability to cause cells to shift from the use of glucose to the use of amino acids as an energy source has significant implications for the use of transient expression-stabilizing compounds. For example, the tricarboxylic acid cycle by which amino acids are metabolized is critical also in the metabolism of fats and lipids. Thus, treating cells or a human subject with transient expression-inducing compounds may result also in the increased metabolism of fats and lipids by virtue of activating the tricarboxylic acid cycle. Thus, the compounds could serve, for example, as agents for controlling weight. These results also illustrate an association between the unique metabolic signature seen in FIGURES 5A-5C and the physiological state in which the transient expression of transfected genes is enhanced and stabilized.

To prepare nucleic acids, 2.8×10^5 trypsinized cells harvested at the end of the 32-day incubation were pelleted by centrifugation, washed with 5 ml of calcium-free and magnesium-free PBS, and mixed with 1 ml of TRIZOL™ (Life Technologies) reagent at room temperature. The cells suspended in TRIZOL™ were then incubated at 4°C for ten minutes. At this point, the sample was stored frozen at -70°C. After being thawed, the sample was permitted to stand at room temperature for 20 minutes before adding 200 µl of chloroform, mixing vigorously for 15 seconds, and incubating at room temperature for 5-20 minutes. Next, the sample was centrifuged at 2,000 x g for 15 minutes at 4°C to separate the emulsion into two phases.

For isolating RNA, the upper aqueous phase was carefully collected without including any portion of the interphase, and transferred to another tube to precipitate the RNA, 0.5 ml of isopropanol was mixed with this aqueous phase, the tube was incubated at room temperature for 10-20 minutes, and then was centrifuged at 12,000 x g at 4°C to collect the RNA pellet. The pellet was carefully washed with 1 ml of 70% (v/v) ethanol, air-dried for 5-10 minutes at room temperature, and resuspended in 30 µl of RNAse-free water (Five Prime Three Prime).

To isolate the DNA, the lower phase and organic layers described above were collected and mixed by inversion with 300 µl of 100% ethanol, then allowed to stand at room temperature for 2-3 minutes to precipitate the DNA. The DNA pellet was collected by centrifugation at 2,000 x g for five minutes at 4°C, then washed twice with 0.1 M sodium citrate containing 10% ethanol. After the second wash, the DNA pellet was again collected by centrifugation at 2,000 g for five minutes at 4°C, and washed by being resuspended in 75% ethanol for 10-20 minutes at room temperature. The pellet was again collected by centrifugation, briefly dried, and resuspended and dissolved in 8 mM sodium hydroxide.

To detect the presence of β-galactosidase sequences, the concentration of the RNA was determined by reading the absorbence at 260 nm, then the RNA solution was diluted with RNAse-free water to a final concentration of 100 µg/ml. Fifty µl of the diluted RNA solution was then mixed with 150 µl of a 50:50 solution of 37% formaldehyde and 20 x SSC. Samples were heated to 55-60°C for 20 minutes to denature the target nucleic acid, placed on ice, and 200 µl RNA-free water were added. Samples were shaken and briefly centrifuged to pellet debris, then loaded into the wells of a slot-blot apparatus under light vacuum to collect the RNA onto a GeneScreen Plus™ membrane (New England Nuclear). Wells were washed with 50 µl of 10 x SSC, and the membrane was exposed to ultraviolet light to crosslink the RNA to the membrane, then was baked for one hour at about 90°C to remove the formaldehyde. DNA samples were slot-blotted using the same procedure, except no vacuum was used.

The presence of β-galactosidase DNA or mRNA on the slot-blot membranes was determined by hybridization with a ^{32}P-labeled oligonucleotide corresponding to a portion of the β-galactosidase gene present in the plasmid used for transfection. The nucleotide sequence of this oligonucleotide was 5' CTCCAACGCAGCACCATCAC 3' (SEQ ID NO:1). For hybridization, 10 ml of hybridization buffer (1 ml 50 x Denhardt's solution, 10 µl of 10 mg/ml polyadenylic

acid, 12.5 ml of 20 x SSC, 5 ml of 10% sodium dodecyl sulfate, and 2.5 ml of 0.5 M $NaPO_4$ (pH 6.5) in a final volume of 50 ml) were placed in a plastic bag with the loaded slot-blot membrane and 1 x 10^6 counts/ml of ^{32}P-labeled probe. Bags were sealed and incubated overnight at 52-53°C. After hybridization, the membranes were washed twice with buffer containing 5 x SSC and 0.1% sodium dodecyl sulfate for 5-10 minutes at room temperature, then twice more with the same buffer at 52-53°C for 20-30 minutes per wash, then exposed to x-ray film.

On the resulting autoradiograms, a signal was present indicating the presence of transfected DNA containing the β-galactosidase gene in the cells harvested 32 days after transfection. Thus, the DNA evidently had persisted in relatively high amounts throughout the 32 day test period. Also, the autoradiogram of the RNA slot-blot showed a surprisingly strong signal after hybridization with the β-galactosidase probe. In numerous previous experiments, it was shown that production of β-galactosidase declined and disappeared from cells within 2-3 days after removing the inducing compounds from the culture medium. Thus, it was clear that the observed persistence of detectable β-galactosidase DNA and mRNA in this experiment did not result from the outgrowth of cells in which the foreign DNA had become integrated. Moreover, the typical half-life for an mRNA is only about 1-3 days, thus the presence of β-galactosidase mRNA at the end of the 32-day incubation period suggests that this mRNA was recently transcribed and that the transfected foreign DNA thus must have persisted throughout the 32-day experiment.

The detection of β-galactosidase DNA after 32 days of incubation suggests furthermore that the foreign DNA may have replicated and increased in amount during this period. Because the cells continued to grow and divide during the experiment, one would have expected the plasmid DNA added at day 0 to have become diluted, and therefore that cells analyzed 32 days later would contain very little β-galactosidase DNA. Thus, the surprising presence of easily detectable amounts of β-galactosidase mRNA and DNA suggests that the transfected DNA may have replicated during the experiment, possibly within the mitochondria.

Example 9.

Induction of Alkaline Phosphatase in Cells Treated with Transient-Expression Stabilizing Compounds

The results of the following experiment indicated that, in addition to inducing the tricarboxylic acid cycle, the metabolic signature of cells treated as described in Example 8 also includes the induction of an endogenous alkaline phosphatase activity

that normally is barely detectable in SW480 cells. Cells were grown in plastic tissue culture dishes, and were transfected and propagated using the same culture media described in Example 8. The cells were fed every few days by the addition of a few ml of feeding medium. Aliquots of the culture medium from these plates were harvested daily for 14 days, beginning with the first day post-transfection, and analyzed for concentrations of glucose, lactate, and ammonia as described in Example 8. Unexpectedly, when these same samples were analyzed for endogenous alkaline phosphatase activity, high amounts were found to be present. The observed degree of elevation ranged from about 2-fold to about 20-fold, as compared with conventionally-grown SW480 cells.

The assay used to measure the SEAP activity was as follows. One-half ml of each sample was mixed with 2 x SEAP buffer (1 x SEAP buffer = 1 M diethanolamine, 0.50 mM magnesium chloride, pH 9.8). As a control, bovine intestinal mucosal alkaline phosphatase was assayed concurrently. The bovine alkaline phosphatase was made up in 1 x SEAP. The chromogenic substrate for these assays was 0.15 M p-nitrophenylphosphate which yields a product detectable at 405 nm after being cleaved by alkaline phosphatase. The substrate (100 µl) was added to each assay tube, then the tubes were placed at 37°C. Thereafter, the absorbance of the control sample was read each minute for 10 minutes, and that of each test sample at 1 and 6 minutes. The units of alkaline phosphatase/ml of test sample were determined using the formula:

$$\text{units enzyme/ml} = \frac{\left[\left(\frac{\Delta A\,405nm}{min}\right)sample - \left(\frac{\Delta A\,405nm}{min}\right)blank\right] V \times df}{18.5 \times VE,}$$

where:

A405nm = absorbance at 405nm,

V = volume in the assay tube,

df = dilution factor,

VE = volume of sample added to the assay tube.

For this set of assays, V=1.1 ml, df=2.2, and VE=0.5 ml.

To determine whether the induced alkaline phosphatase activity was heat-sensitive, a second set of assays was run on the same samples using assay buffer identical to the SEAP buffer described above, but containing 0.01 M L-homoarginine. The control enzyme samples and samples of culture medium were heated in this buffer to 65°C for 5-10 minutes before adding the substrate. This heat treatment is known

to destroy the alkaline phosphatase that is found in most mammalian cells that express the enzyme. This pre-treatment indeed destroyed the alkaline phosphatase activity in these samples, as well as that in the control enzyme samples, thus indicating that the induced alkaline phosphatase corresponded to the type of alkaline phosphatase most commonly detected in animal cells, rather than to the heat-resistant variety known to be present in placenta.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

SEQUENCE LISTING

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(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: oligonucleotide
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: YES
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCCAACGCA GCACCATCAC 20

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

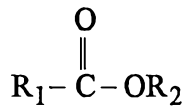
1. A method of enhancing the transient expression of a foreign gene in a eukaryotic cell comprising:

introducing into the cell a molecule of foreign DNA that encodes a protein in a form capable of being expressed in the cell; and,

contacting the cell before, during, or after introducing the DNA with a transient expression enhancing agent.

2. The method of Claim 1, wherein said agent induces the cell to use proteins or amino acids as its primary energy source.

3. The method of Claim 1, wherein the transient expression enhancing agent comprises at least one carboxylic acid derivative having the formula:



wherein R_1 is:

$CHNH_2R_3$, wherein R_3 is the side chain of a naturally occurring amino acid;

$C_6H_4R_4$, wherein R_4 is H, CH_3, $(CH_2)_nCH_3$, NH_2, $COCH_3$, $CO(CH_2)_nCH_3$, $C(CH_3)_3$, $CH(CH_3)_2$, $(CH_2)_nCH(CH_3)_2$, $(CH_2)_nCOCH_3$, OCH_3, or $O(CH_2)_nCH_3$, wherein n = 1-3;

$CHNH_2(CH_2)_nR_5$, wherein n = 1-7 and R_5 is CH_3, OH, $CONH_2$, C_6H_4OH, or $CONHNH_2$;

$(CH_2)_nR_6$, wherein n = 1-9 and R_6 is an indole group, $NCH_3C(=NH)NH_2$, SCH_3, NH_2, CH_3, CO_2H, $CONH_2$, or $NHC(=NH)NH_2$;

$(CH_2)_nCHNH_2CO_2H$, wherein n = 1-8;

$CH(CO_2H)NHCONH_2$;

C_5H_4N; and

wherein R_2 is selected from H, CH_3, $(CH_2)_nCH_3$ wherein n = 1-8, $(CH_2)_xO(CH_2)_yCH_3$ or $(CH_2)_xCO(CH_2)_yCH_3$ wherein x + y = 2-7, or M, wherein M is a metal counterion or a low molecular weight organic cation.

4. The method of Claim 3 wherein the transient expression enhancing agent comprises an amino acid derivative selected from the group consisting of 3-methyl-L-histidine, α-ketoglutaric acid, β-alanine, carnosine, citrulline, creatine, folic acid, glutathione, hippuric acid, homoserine, N-(4-aminobenzyl)-L-glutamic diethylester, N-carbamyl aspartic acid, N-formyl-L-methionine, and ornithine.

5. The method of Claim 3, wherein R_1 is non-polar and hydrophobic.

6. The method of Claim 1, wherein the transient expression enhancing agent comprises a sulfonic acid derivative having the formula:

$$R_7\text{-}SO_2\text{-}OR_8$$

wherein R_7 is a lower alkyl, aryl, substituted lower alkyl, aryl, substituted lower alkyl, or substituted aryl; and

R_8 is a hydrogen, a metal counterion, or a low molecular weight organic cation.

7. The method of Claim 6 wherein R_7 is an amino substituted lower alkyl group or an amino substituted aryl group.

8. The method of Claim 6 wherein the sulfonic acid derivative is selected from the group consisting of 3-aminobenzene sulfonic acid, taurine, and salts thereof.

9. The method of Claim 1, wherein the transient expression enhancing agent comprises a sulfonated amino polysaccharide.

10. The method of Claim 9, wherein the sulfonated amino polysaccharide comprises an N-acetylated amino polysaccharide.

11. The method of Claim 10, wherein the N-acetylated amino polysaccharide is selected from the group consisting of chondroitin-6-sulfate and a guaran.

12. The method of Claim 10 wherein the guaran is a hydroxypropyl guaran.

13. The method of Claim 1 wherein the transient expression enhancing agent comprises a compound selected from the group consisting of adrenaline, coenzyme B12, and methylcobalamin.

14. The method of Claim 3, wherein the concentration of the transient expression enhancing agent is 1-15 mM.

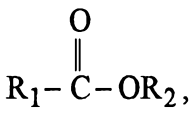
15. The method of Claim 6, wherein the concentration of the transient expression enhancing agent is 1-15 mM.

16. The method of Claim 9, wherein the concentration of the transient expression enhancing agent is 0.01-0.5 mM.

17. The method of Claim 11, wherein the N-acetylated amino polysaccharide is chondroitin-6-sulfate having a molecular weight of about 4000 daltons.

18. The method of Claim 1, wherein the transient expression enhancing agent comprises an aqueous solution having a pH of 4.5-9.0.

19. The method of Claim 1, wherein the cell is contacted with a first transient expression enhancing agent prior to, during, and following the introduction into the cell of the foreign DNA, wherein the agent comprises at least one compound having the formula:



wherein R_1 is:

$CHNH_2R_3$, wherein R_3 is the side chain of a naturally occurring amino acid;

$C_6H_4R_4$, wherein R_4 is H, CH_3, $(CH_2)_nCH_3$, NH_2, $COCH_3$, $CO(CH_2)_nCH_3$, $C(CH_3)_3$, $CH(CH_3)_2$, $(CH_2)_nCH(CH_3)_2$, $(CH_2)_nCOCH_3$, OCH_3, or $O(CH_2)_nCH_3$, wherein n = 1-3;

$CHNH_2(CH_2)_nR_5$, wherein n = 1-7 and R_5 is CH_3, OH, $CONH_2$, C_6H_4OH, or $CONHNH_2$;

$(CH_2)_nR_6$, wherein n = 1-9 and R_6 is an indole group, $NCH_3C(=NH)NH_2$, SCH_3, NH_2, CH_3, CO_2H, $CONH_2$, or $NHC(=NH)NH_2$;

$(CH_2)_nCHNH_2CO_2H$, wherein n = 1-8;

$CH(CO_2H)NHCONH_2$;

C_5H_4N; and

wherein R_2 is H, CH_3, $(CH_2)_nCH_3$ wherein n = 1-8, or $(CH_2)_xO(CH_2)_yCH_3$ or $(CH_2)_xCO(CH_2)_yCH_3$ wherein x + y = 2-7, or M, wherein M is a metal counterion or a low molecular weight organic cation;

or the first transient expression enhancing agent comprises a compound having the formula:

$$R_7\text{-}SO_2\text{-}OR_8,$$

wherein R_7 is a lower alkyl, aryl, substituted lower alkyl, or substituted aryl; and

R_8 is a hydrogen, a metal counterion, or a low molecular weight organic cation;

and following the introduction of the foreign DNA, the cell is contacted with a second transient expression enhancing agent, wherein the second agent comprises a sulfonated amino polysaccharide.

20. The method of Claim 1, wherein the cell is contacted with the agent prior to introduction of the foreign DNA into the cell.

21. The method of Claim 1, wherein the cell is contacted with the agent during the introduction of the foreign DNA into the cell.

22. The method of Claim 1, wherein the cell is continuously exposed to the agent after the introduction of the foreign DNA into the cell.

23. The method of Claim 1, wherein the cell is a cultured cell.

24. The method of Claim 23, wherein the cultured cell is a primary culture.

25. The method of Claim 24, wherein the cultured cell is selected from the group consisting of a stably transformed cell, a tumor cell line, and a hybridoma cell.

26. The method of Claim 25, wherein the cultured cell is a SW480 P3 cell.

27. The method of Claim 1, wherein the agent is biocompatible.

28. The method of Claim 1, wherein the protein encoded by the foreign gene is harvested.

29. The method of Claim 1, wherein the cell is present in a live host, and the transient expression enhancing agent is introduced into the host orally or by injection.

30. The method of Claim 1, wherein the foreign DNA is introduced into the cell by a method selected from the group consisting of lipofection, a viral vector, exposure of cells to coprecipitates of calcium phosphate, and transfection in the presence of starburst polymers.

31. The method of Claim 30, wherein the DNA is introduced into the cell by a viral vector, and the viral vector is derived from an adenovirus.

32. The method of Claim 27, wherein the agent contains at least one hydrophobic moiety and at least one acidic moiety, and wherein the acidic group may be modified to form a salt or an ester.

33. The method of Claim 32, wherein the acidic moiety is hydrophobic and organic.

34. A method of screening an agent comprising at least one chemical compound to determine whether the agent is capable of enhancing the transient expression of a foreign gene in a eukaryotic cell, wherein the agent is biocompatible and contains at least one hydrophobic moiety and at least one acidic moiety, the method comprising the steps of:

introducing into a first and a second SW480 P3 cell on day 0 a molecule of foreign DNA that encodes a protein in a form capable of being expressed in the cells;

before, during, or after introducing the DNA, contacting the second cell with the agent;

cumulatively measuring in both cells between days 0 and 4, or between days 4 and 7, or between days 4 and 14 the amount of protein expressed from the foreign DNA, and using these amounts to determine, respectively, a value for X, G_7, or G_{14} according to the formula:

$$X, \text{ or } G_7 \text{ or } G_{14} = 100 - \frac{(A \times 100)}{C},$$

wherein "A" is the amount of the protein encoded by the foreign gene expressed in the first cell and, "C" is the amount of protein expressed in the second cell; and,

determining that the agent is capable of enhancing transient expression if X or G_7 or G_{14} is greater than 10.

35. The method of Claim 34, wherein X or G_7 or G_{14} is greater than 25.

36 A method of enhancing the transient expression of a foreign gene in a cell comprising:

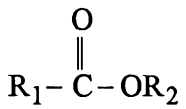
introducing into the cell a molecule of foreign DNA that encodes a protein in a form capable of being expressed in the cell; and,

contacting the cell with an agent for which X or G_7 or G_{14} is greater than 25 when the agent is evaluated according to the assay of Claim 33.

37. A method of manipulating the metabolism of a cell to reduce the cell's consumption of glucose, comprising the step of contacting the cell with an agent that induces the cell to use proteins or amino acids as their primary energy source.

38. The method of Claim 37, wherein the agent further induces the cell to express an endogenous alkaline phosphatase activity.

39. The method of Claim 37, wherein said agent is capable of enhancing the transient expression of a foreign gene in the cell, wherein the agent comprises at least one chemical compound having the formula:



wherein R_1 is:

$CHNH_2R_3$, wherein R_3 is the side chain of a naturally occurring amino acid;

$C_6H_4R_4$, wherein R_4 is H, CH_3, $(CH_2)_nCH_3$, NH_2, $COCH_3$, $CO(CH_2)_nCH_3$, $C(CH_3)_3$, $CH(CH_3)_2$, $(CH_2)_nCH(CH_3)_2$, $(CH_2)_nCOCH_3$, OCH_3, or $O(CH_2)_nCH_3$, wherein n = 1-3;

$CHNH_2(CH_2)_nR_5$, wherein n = 1-7 and R_5 is CH_3, OH, $CONH_2$, C_6H_4OH, or $CONHNH_2$;

$(CH_2)_nR_6$, wherein n = 1-9 and R_6 is an indole group, $NCH_3C(=NH)NH_2$, SCH_3, NH_2, CH_3, CO_2H, $CONH_2$, or $NHC(=NH)NH_2$;

$(CH_2)_nCHNH_2CO_2H$, wherein n = 1-8;

$CH(CO_2H)NHCONH_2$;

C_5H_4N; and

wherein R_2 is H, CH_3, $(CH_2)_nCH_3$ wherein n = 1-8, $(CH_2)_xO(CH_2)_yCH_3$ or $(CH_2)_xCO(CH_2)_yCH_3$ wherein x + y = 2-7, or M, wherein M is a metal counterion or a low molecular weight organic cation; or

the group consisting of a sulfonic acid derivative having the formula:

$$R_7\text{-}SO_2\text{-}OR_8$$

wherein R_7 is a lower alkyl, aryl, substituted lower alkyl, or substituted lower aryl; and

R_8 is a hydrogen atom, a metal counterion, or a low molecular weight organic cation; or

a sulfonated amino polysaccharide.

40. The method of Claim 39, wherein the agent comprises a chemical compound selected from the group consisting of benzoic acid, 4-ethylbenzoic acid, benzoate buffer, and chondroitin-6-sulfate.

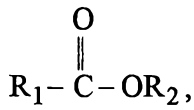
41. The method of Claim 39, wherein the agent is administered *in vivo* to a mammal.

42. A method of enhancing the adhesion of a cell to a culture substratum, wherein a sulfonated amino polysaccharide is added to the medium in which the cells are cultured.

43. The method of Claim 42, wherein the cell is a cell that normally requires a feeder layer in order to grow in culture.

44. The method of Claim 42, wherein the cell is a hepatocyte, and the sulfonated amino polysaccharide is chondroitin-6-sulfate.

45. The method of Claim 1, wherein the cell is contacted with a first agent prior to, during, and following the introduction into the cell of the foreign DNA, wherein the first agent comprises at least one chemical compound having the formula:



wherein R_1 is:

$CHNH_2R_3$, wherein R_3 is the side chain of a naturally occurring amino acid;

$C_6H_4R_4$, wherein R_4 is H, CH_3, $(CH_2)_nCH_3$, NH_2, $COCH_3$, $CO(CH_2)_nCH_3$, $C(CH_3)_3$, $CH(CH_3)_2$, $(CH_2)_nCH(CH_3)_2$, $(CH_2)_nCOCH_3$, OCH_3, or $O(CH_2)_nCH_3$, wherein n = 1-3;

$CHNH_2(CH_2)_nR_5$, wherein n = 1-7 and R_5 is CH_3, OH, $CONH_2$, C_6H_4OH, or $CONHNH_2$;

$(CH_2)_nR_6$, wherein n = 1-9 and R_6 is an indole group, $NCH_3C(=NH)NH_2$, SCH_3, NH_2, CH_3, CO_2H, $CONH_2$, or $NHC(=NH)NH_2$;

$(CH_2)_nCHNH_2CO_2H$, wherein n = 1-8;

$CH(CO_2H)NHCONH_2$;

C_5H_4N; and

wherein R_2 is H, CH_3, $(CH_2)_nCH_3$ wherein n = 1-8, $(CH_2)_xO(CH_2)_yCH_3$ or $(CH_2)_xCO(CH_2)_yCH_3$ wherein x + y = 2-7, or M, wherein M is a metal counterion or a low molecular weight organic cation;

or the first agent comprises at least one chemical compound having the formula:

$$R_7\text{-}SO_2\text{-}OR_8,$$

wherein R_7 is a lower alkyl, aryl, substituted lower alkyl, or substituted lower aryl; and

R_8 is a hydrogen atom, a metal counterion, or a low molecular weight organic cation;

and following the introduction of the foreign DNA, the cell is contacted with a second agent, wherein the second agent comprises at least one sulfonated amino polysaccharide or wherein the second agent comprises at least one chemical compound having the formula:

$$R_1-\overset{\overset{O}{\|}}{C}-OR_2,$$

wherein R_1 is:

$CHNH_2R_3$, wherein R_3 is the side chain of a naturally occurring amino acid;

$C_6H_4R_4$, wherein R_4 is H, CH_3, $(CH_2)_nCH_3$, NH_2, $COCH_3$, $CO(CH_2)_nCH_3$, $C(CH_3)_3$, $CH(CH_3)_2$, $(CH_2)_nCH(CH_3)_2$, $(CH_2)_nCOCH_3$, OCH_3, or $O(CH_2)_nCH_3$, wherein n = 1-3;

$CHNH_2(CH_2)_nR_5$, wherein n = 1-7 and R_5 is CH_3, OH, $CONH_2$, C_6H_4OH, or $CONHNH_2$;

$(CH_2)_nR_6$, wherein n = 1-9 and R_6 is an indole group, $NCH_3C(=NH)NH_2$, SCH_3, NH_2, CH_3, CO_2H, $CONH_2$, or $NHC(=NH)NH_2$;

$(CH_2)_nCHNH_2CO_2H$, wherein n = 1-8;

$CH(CO_2H)NHCONH_2$;

C_5H_4N; and

wherein R_2 is H, CH_3, $(CH_2)_nCH_3$ wherein n = 1-8, $(CH_2)_xO(CH_2)_yCH_3$ or $(CH_2)_xCO(CH_2)_yCH_3$ wherein x + y = 2-7, or M, wherein M is a metal counterion or a low molecular weight organic cation;

or the second agent comprises at least one chemical compound having the formula:

$$R_7\text{-}SO_2\text{-}OR_8,$$

wherein R_7 is a lower alkyl, aryl, substituted lower alkyl, or substituted lower aryl; and

R_8 is a hydrogen atom, a metal counterion, or a low molecular weight organic cation; and

wherein the first agent is one that has a value for X that is greater than 25 when X is calculated according to the formula:

$$X = 100 - \frac{(A \times 100)}{C}$$

and wherein the second agent is one that has a value of G_7 or G_{14} that is greater than 25, wherein G_7 or G_{14} is calculated according to the formula:

$$G_7 \text{ or } G_{14} = 100 - \frac{(A \times 100)}{C}$$

wherein for both for both X and G_7 or G_{14}, "A" is the amount of the protein encoded by the foreign gene expressed in a first cell that is contacted with the first and the second agent, and "C" is the amount of protein expressed in a second cell that is not contacted with the first or the second agent.

46. The method of Claim 45, wherein the first agent is benzoate buffer, and the second agent is chondroitin-6-sulfate.

47. The method of Claim 45, wherein the first agent comprises benzoic acid and 4-ethylbenzoic acid, and the second agent comprises benzoate buffer and chondroitin-6-sulfate.

48. The method of Claim 45, wherein the first agent comprises benzoate buffer and glutamic acid, and the second agent comprises chondroitin-6-sulfate.

49. The method of Claim 45, wherein the cell is contacted with the first agent for about 20-24 hours prior to the introduction into the cell of the foreign DNA.

50. The method of Claim 1, wherein the agent is: benzoic acid and 4-ethylbenzoic acid; or benzoate buffer and chondroitin-6-sulfate; or benzoate buffer and glutamic acid; or glutathione, methionine, glycine, α-amino-n-butyric acid, taurine, phenylalanine, benzoate buffer, and alanine.

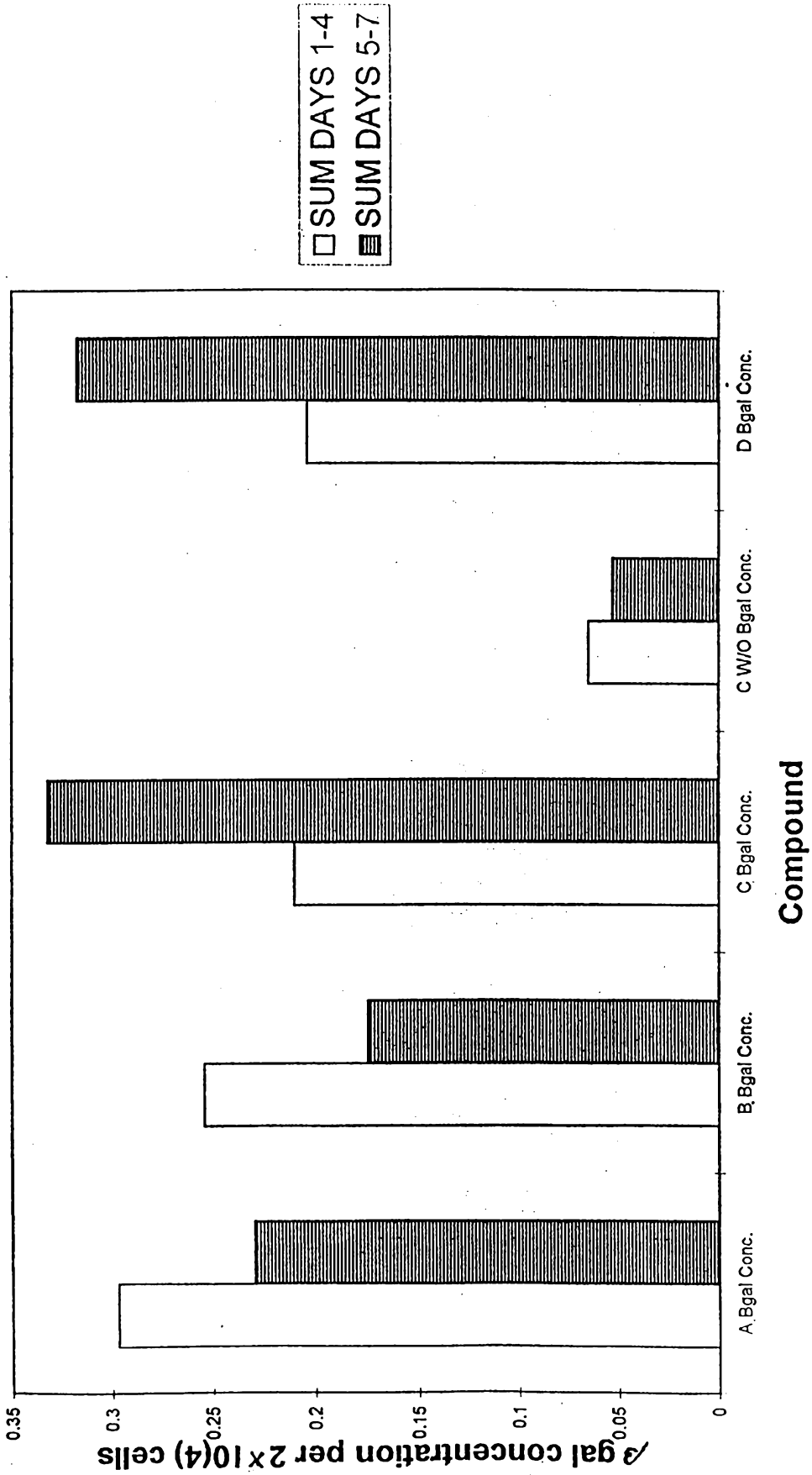

SUM DAYS 1-4
SUM DAYS 5-7
β gal concentration per 2 x 10(4) cells
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
A Bgal Conc.
B Bgal Conc.
C Bgal Conc.
C W/O Bgal Conc.
D Bgal Conc.
Compound

Figure 1

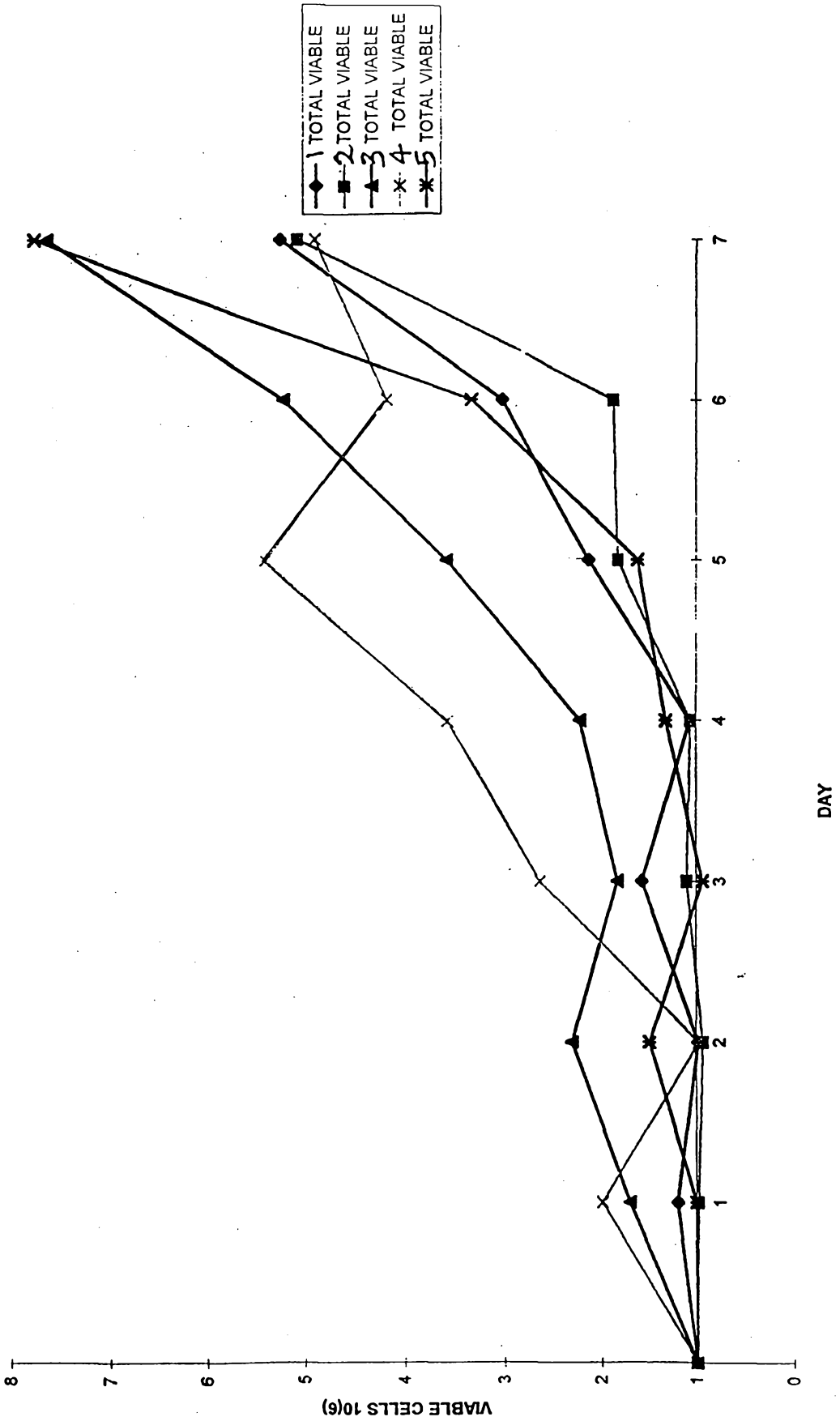

1 TOTAL VIABLE
2 TOTAL VIABLE
3 TOTAL VIABLE
4 TOTAL VIABLE
5 TOTAL VIABLE
VIABLE CELLS 10(6)
0
1
2
3
4
5
6
7
8
DAY
1
2
3
4
5
6
7

FIGURE 2

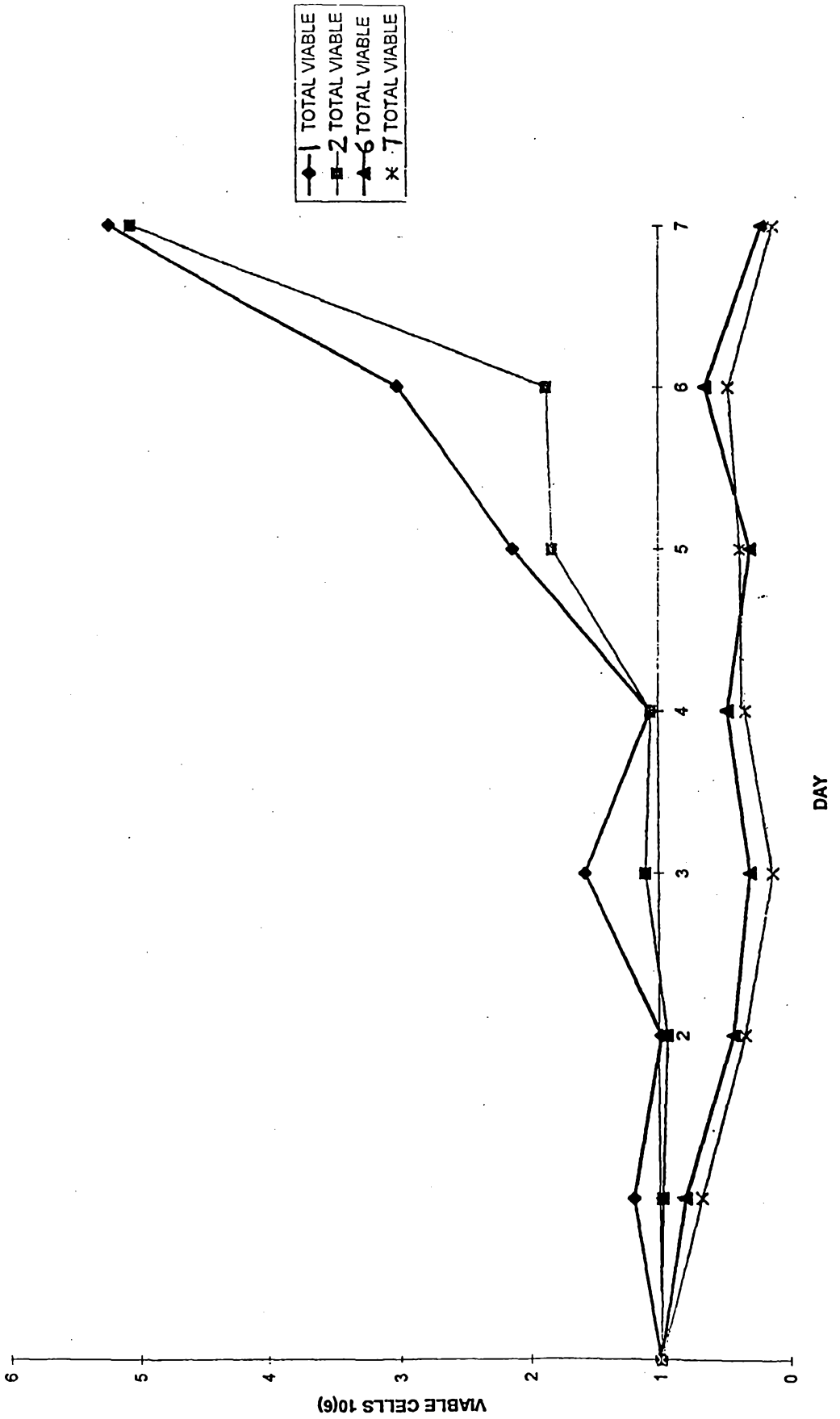
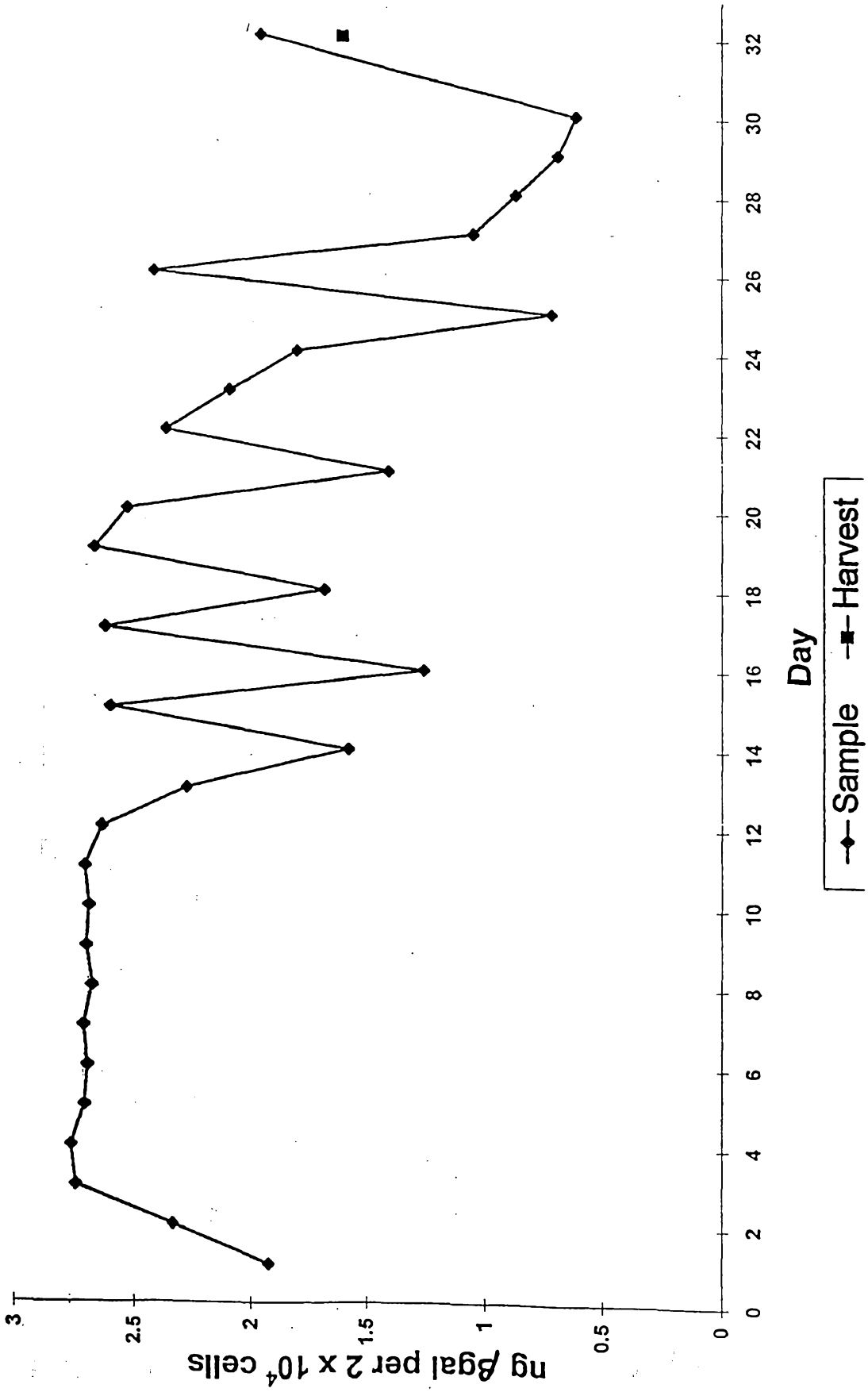

1 TOTAL VIABLE
2 TOTAL VIABLE
6 TOTAL VIABLE
7 TOTAL VIABLE
VIABLE CELLS 10(6)
DAY
0
1
2
3
4
5
6
7

FIGURE 3

Fig. 4


ng βgal per 2 x 10⁴ cells
0
0.5
1
1.5
2
2.5
3
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
Day
Sample
Harvest

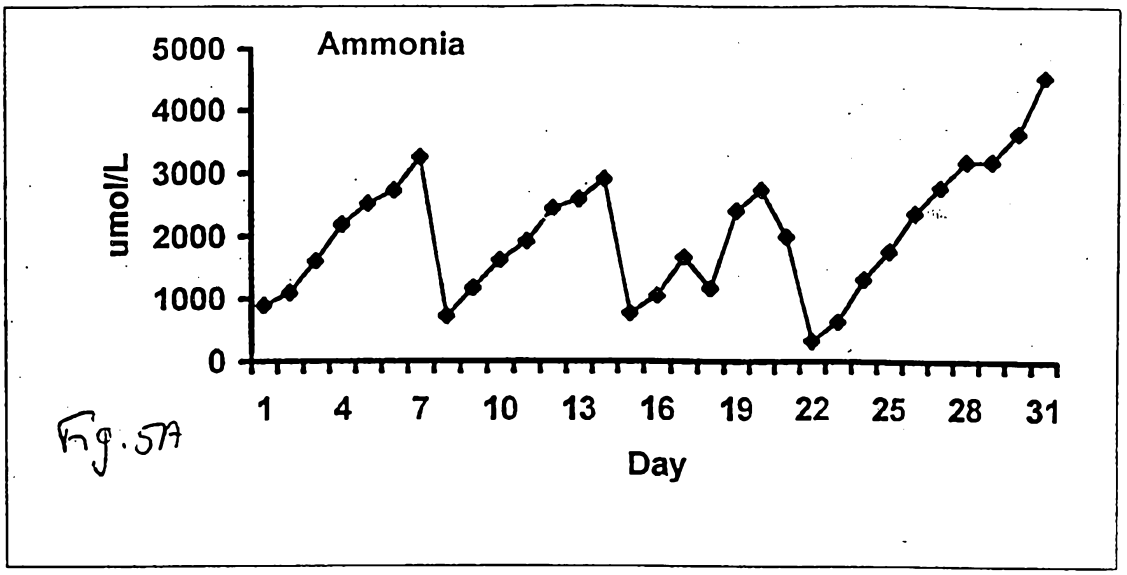


Fig. 5A

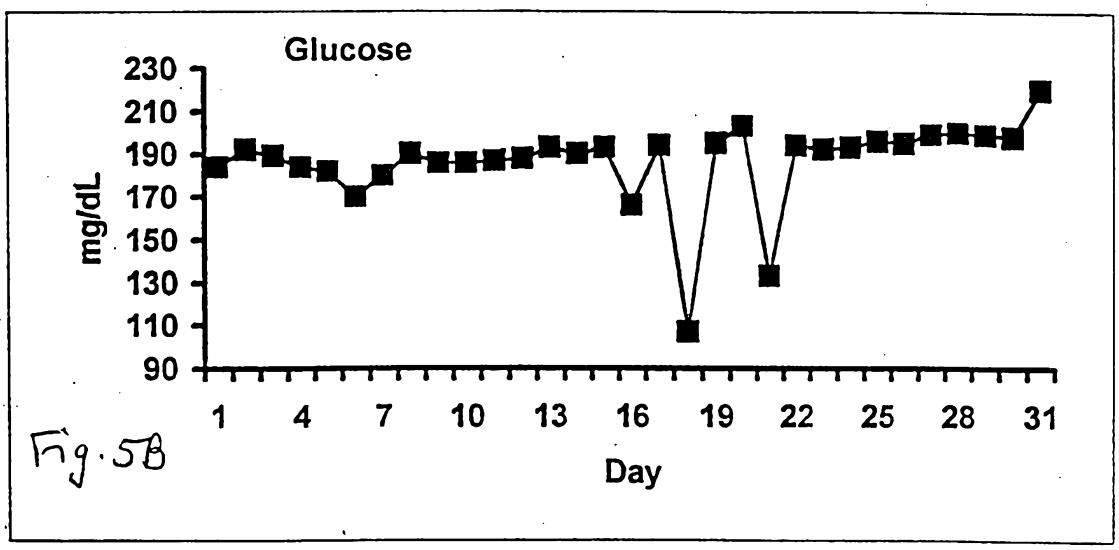


Fig. 5B

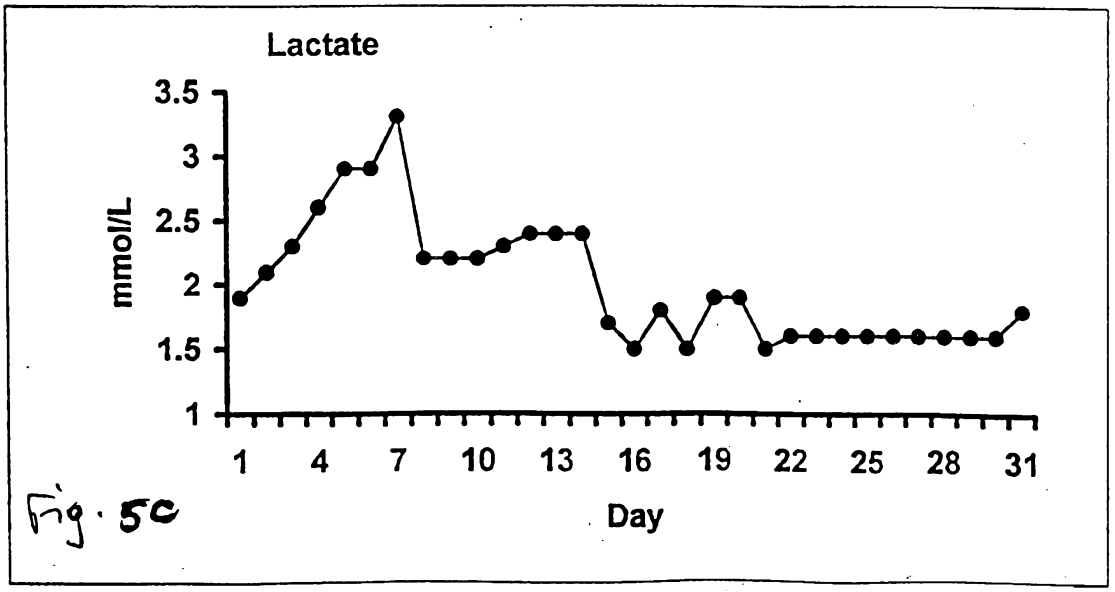


Fig. 5C