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(71) Applicant (for all designated States except US): **UNIVERSITY OF MARYLAND, BALTIMORE** [US/US]; 520 West Lombard Street, Baltimore, MD 21201-1627 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **ZHAO, Richard, Y.** [US/US]; 21801 Gaithers Meadow Lane, Brookeville, MD 20833 (US). **ELDER, Robert, T.** [US/US]; 1528 E. 59th Street, Apt. 1F, Chicago, IL 60637 (US).

(74) Agent: **SISTRUNK, Melissa, L.**; Fulbright & Jaworski L.L.P., Fulbright Tower, 1301 McKinney, Suite 5100, Houston, TX 77010-3095 (US).

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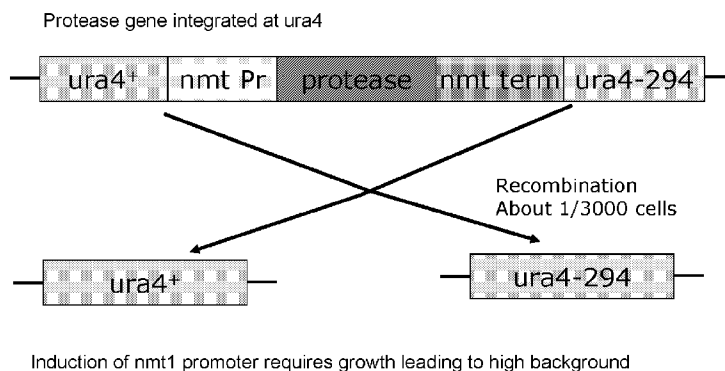
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(54) Title: SYSTEM AND COMPOSITIONS FOR ISOLATING SUPPRESSORS OF HIV-1 PROTEASE

Loss of integrated gene by homologous recombination creates a high background in suppressor screens



(57) Abstract: The present invention relates to a yeast screening assay for the identification of suppressors of a protease, such as the HIV-I protease and/or HIV-2 protease, for example. Upon overexpression of HIV-I protease in *Schizosaccharomyces pombe*, for example, the cell dies, but in the presence of a candidate inhibitor with protease suppressor activity, the cell lives, thereby providing an assay for the identification of one or more suppressors. In additional aspects, there is an in vivo protease activity screen, used alternatively or additionally to a viability screen, such as for cleavage of a HIV-I protease-specific site. In further aspects, the suppressor is employed for the treatment of an individual infected with HIV virus or with AIDS.

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SYSTEM AND COMPOSITIONS FOR ISOLATING SUPPRESSORS OF HIV-1 PROTEASE

[0001] The present invention claims priority to U.S. Provisional Patent Application Serial No. 60/812,685, filed June 9, 2006, which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] The invention may have been developed with funds from the National Institutes of Health Grant Nos. NIH NIGM R01 GM63080 and N1H-N1AID R01 AI40891. The United States Government has certain rights in the invention.

TECHNICAL FIELD

[0003] The present invention relates at least to the fields of cell biology, molecular biology, and medicine. In particular, the present invention relates to the field of pharmaceuticals for HIV/AIDS therapy.

BACKGROUND OF THE INVENTION

[0004] HIV/AIDS is still an incurable disease that affects million of lives world-wide. The cocktail therapy, also known as HAART or ART, is currently the most successful antiretroviral strategy to reduce HIV-1 to undetectable levels in infected patients. Typical ART requires the use of a HIV-1 protease inhibitor (PI) in combination with two HIV-1 reverse transcriptase inhibitors. Among the two classes of anti-HIV regimens, the HIV-1 PIs are the most potent viral inhibitors that in monotherapy can reduce viral load 2-3 logs. The HIV protease is essential for viral infectivity and cleaves the viral polyprotein (gag-pol) into active viral enzymes (reverse transcriptase, protease and integrase) and structural proteins. Thus, the HIV-1 protease is required for the production of structural proteins and enzymes of virus as well as for maturation of infectious viral particles. At present, there are a total of 8 PIs which have been approved by FDA; all of these are competitive inhibitors which bind at the active site of the protease. Many of these PI were in fact designed to fit the structure of the active site. This binding of the PI at the active site prevents the protease from cleaving the viral precursor polypeptide and blocks subsequent viral maturation. One of the major challenges in ART is the

rapid emergence of viral drug resistance. Viral resistance to PIs generally emerges by stepwise accumulation of mutations in the protease gene and is growing rapidly. Failure of ART can occur as the result of multi-drug resistance where the protease becomes resistant to most or all of the PI being used, raising an alarming possibility that HIV multi-drug resistance may ultimately outgrow the number of PIs that are currently available. Therefore, there is an urgent need to develop additional PI that is active against these resistant proteases.

SUMMARY OF THE INVENTION

[0005] The present invention is directed to a system, methods, and compositions that relate to screens for HIV therapeutics and uses of the therapeutics.

[0006] In particular, there are certain advances in the art, achieved by the inventors, that lead to the development and use of exemplary methods to screen for suppressors of the HIV-1 protease, although in alternative embodiments HIV-2 protease is employed. In a first embodiment, high level expression of the protease gene kills fission yeast. This observation provides the basis for identifying protease suppressors that can be isolated by their ability to allow growth when the protease gene is expressed.

[0007] The exemplary cell-based screening described herein identifies compounds that inhibit both the wild type and resistant proteases, in particular embodiments. These new inhibitors in certain cases might not bind at the active site and could thus reveal new ways to inhibit the protease by targeting other regions and properties of the protease. In specific cases, the invention provides several fission yeast cell-based assays for use in automated high throughput molecular screen (HTS) for broad spectrum inhibitors of the HIV-1 protease. These assays may be employed in 96-well formats and further miniaturized, in certain cases. In one assay for protease activity, there are absorbance measurements of cell density, and in some cases there is or is also a fluorescence measurement of cell viability, for example as a secondary confirming assay. In an additional or alternative case, there is green fluorescence relocalization of GFP-substrate-Vpr fusion protein as counter-screen assay for protease activity. These three assays may be configured into HTS format and/or may employ a library of potential inhibitors, for example a small Sigma LOPAC1280 compound library, in specific embodiments.

[0008] In certain aspects of the invention, there are more than one levels to the screen to identify suppressors of HIV-1 protease. For example, the screen may employ viability as one layer of screen to identify those suppressors that allow growth to occur when the protease

is expressed. In another level of the screen, which may be utilized in conjunction with or in lieu of a viability assay, there is an *in vivo* assay that screens for enzymatic assay by the protease, for example cleavage of a protease substrate. Although in exemplary embodiments the cleavage site can be any suitable site that HIV-1 cleaves, in specific embodiments the cleavage site is comprised in SEQ ID NO:3 or SEQ ID NO:4.

[0009] In another embodiment, there is an *in vivo* assay for protease activity wherein activity is illustrated *via* green fluorescence protein (GFP) localization in the cell. This assay provides a semi-quantitative measurement of the amount of protease activity in the cell and can be used to characterize the substrate specificity of the protease.

[0010] Concerning at least some embodiments of the invention, one aspect to isolating suppressors addresses the background of cells that lose the expressed gene by homologous recombination, for example. By linking a polynucleotide for resistance to an antibiotic, such as resistance to G418, for example, to the protease polynucleotide, these cells with loss of the protease gene can be easily identified and eliminated from the suppressor screen. This approach reduces the background from gene loss to such low levels that it no longer significantly interferes with the screen for protease suppressors.

[0011] Employing an exemplary screen of the invention, the low background and successful use of the procedure to screen a fission yeast genomic library identified the *hhp2* gene of fission yeast as a protease suppressor. There is no previous report known to the inventors of any relationship between *hhp2* and the HIV-1 protease gene, and the mechanism of suppression may be characterized. Therefore, the *hhp2* polynucleotide is one exemplary mechanism of suppression that provides new strategies to inhibit the HIV-1 protease.

[0012] In one embodiment of the invention, there is a method of identifying an HIV protease inhibitor, comprising providing a yeast cell, wherein said yeast cell comprises a polynucleotide comprising sequence encoding HIV protease; subjecting the polynucleotide to a candidate agent; and assessing one or both of the following characteristics: 1) the viability and/or growth of said yeast cell, wherein the ability to survive in the presence of said candidate agent, as compared to in the absence of said candidate agent, identifies said candidate agent as said HIV protease inhibitor; and 2) cleavage of an HIV protease substrate, wherein the ability of the candidate agent to inhibit the cleavage of the substrate identifies said candidate agent as said HIV protease inhibitor. In a specific embodiment, the HIV protease is HIV-1 protease or HIV-2

protease. In another specific embodiment, the expression of the HIV protease is under the regulation of an overexpression regulatory sequence. In a further specific embodiment, the inhibitor is a polypeptide, a polynucleotide, a small molecule, an antibody, or a mixture or combination thereof. The yeast may be a fission yeast, such as *Schizosaccharomyces pombe*, for example.

[0013] In specific embodiments, the polynucleotide is incorporated into the genome of the yeast. The overexpression regulatory sequence comprises a *nmt1* overexpression regulatory sequence, an *adh1* regulatory sequence, a *fbp1* regulatory sequence, an *inv1* regulatory sequence, or a *ctr4* regulatory sequence, in certain cases. The polynucleotide may be further defined as comprising at least one moiety for reducing background growth in a viability screen. In a specific embodiment, the moiety for reducing background comprises a marker, such as a marker that comprises resistance to an antibiotic, a marker that comprises resistance to G418 or hygromycin, a marker that is a nutritional marker, or a marker that comprises *ade1*, *ade6*, *arg3*, *CAN1*, *his3*, *his7*, *leu1*, *leu2*, *sup3-5*, *ura4*, and *ura3*.

[0014] In certain embodiments, the HIV protease is a human HIV protease, and the HIV protease substrate is a human HIV protease substrate. In a specific embodiment, assessment of cleavage of an HIV protease substrate comprises assessing cleavage of a polypeptide comprising a first polypeptide moiety and a second polypeptide moiety, wherein an HIV protease cleavage site is present between said first and second polypeptide moieties. In specific aspects, the first polypeptide moiety comprises a detectable label, and in other specific aspects, the second polypeptide moiety comprises a substrate for HIV protease, such as HIV protease is HIV-1 Vpr polypeptide, for example.

[0015] In a specific embodiment, the cleavage site comprises DSQNYPIVQ (SEQ ID NO:3), DSFNFPQIT (SEQ ID NO:4), VSQNYPIVQN (SEQ ID NO:8), KARVLAEAMS (SEQ ID NO:9), SATIMMQRGN (SEQ ID NO:10), RPGNFLQSRP (SEQ ID NO:11), VSPNFPQITL (SEQ ID NO:12), CTLNFPISPI (SEQ ID NO:13), GAETFYVDG (SEQ ID NO:14), or IRKVLFLDGI (SEQ ID NO:15). In a particular aspect of the invention, a detectable label comprises green fluorescence protein, emerald green fluorescence protein, yellow fluorescence protein, blue fluorescence protein or cyan fluorescence protein. In particular cases, the method further comprises delivering a therapeutically effective amount of said inhibitor to an individual that has been infected with the HIV virus or that has AIDS. In other particular cases,

the method further comprises delivering an effective amount of said inhibitor to an individual at risk for becoming infected with HIV or at risk for developing AIDS.

[0016] In one embodiment, there is a method of treating an individual infected with HIV virus or that has AIDS, comprising the step of delivering to the individual a therapeutically effective amount of a composition comprising hhp2. In a specific case, the hhp2 is a polypeptide, although in other cases, the hhp2 is a polynucleotide encoding a hhp2 polypeptide. An exemplary *S. pombe* hhp2 polynucleotide is provided in SEQ ID NO:16 (GenBank® Accession No. U10864), and an exemplary *S. pombe* hhp2 polypeptide is provided in SEQ ID NO:17 (GenBank® Accession No. AAA21545). In particular embodiments, based on the protein sequence and structure, the fission yeast *hhp2* is a homologue of human casein kinase 1 (CK1); an exemplary CK1 polynucleotide is SEQ ID NO:18 (GenBank® Accession No. X80693), and an exemplary CK1 polypeptide is SEQ ID NO:19 (GenBank® Accession No. CAA56710). Agents of the invention, such as pharmaceutical agents for the treatment of HIV or AIDS, have HIV-1 and/or HIV-2 protease inhibitor function and may comprise SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, or SEQ ID NO:19, for example, or they may comprise molecules having at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 99%, or 100% identity to SEQ ID NO:16 or SEQ ID NO:17.

[0017] In a particular embodiment there is a yeast cell that comprises a polynucleotide encoding HIV protease. In a specific embodiment, the HIV protease is HIV-1 protease or HIV-2 protease. In a specific embodiment of the yeast cell, expression of the polynucleotide is under the control of an overexpression regulatory sequence. In another case, the polynucleotide further comprises a marker, such as a marker for resistance to an antibiotic, or a marker is further defined as a nutritional marker. In an additional case, the yeast cell further comprises a polynucleotide comprising a first sequence encoding a first polypeptide moiety and a second sequence encoding a second polypeptide moiety, wherein a cleavage site for the protease is present between said first and second polypeptide moieties. In another case, the first polypeptide moiety comprises a detectable label, such as green fluorescence protein, emerald green fluorescence protein, yellow fluorescence protein, blue fluorescence protein or cyan fluorescence protein. In particular cases, the second polypeptide moiety comprises HIV-1 Vpr polypeptide. In other aspects, the yeast cell is further defined as being in a culture of yeast cells or a colony of yeast cells.

[0018] In an additional embodiment, there is a kit for the treatment and/or prevention of HIV infection and/or AIDS, comprising a polynucleotide encoding hhp2 or a hhp2 polypeptide housed in a suitable container. In a specific embodiment, the method further comprises a pharmaceutically acceptable excipient. In an additional specific embodiment, there is a kit for screening for one or more HIV protease inhibitors, comprising a yeast cell of the invention. In a specific aspect, the yeast cell further comprises a polynucleotide comprising a first sequence encoding a first polypeptide moiety and a second sequence encoding a second polypeptide moiety, wherein a cleavage site for the protease is present between said first and second polypeptide moieties.

[0019] The foregoing has outlined rather broadly the features and technical advantages of the present invention in order that the detailed description of the invention that follows may be better understood. Additional features and advantages of the invention will be described hereinafter which form the subject of the claims of the invention. It should be appreciated by those skilled in the art that the conception and specific embodiment disclosed may be readily utilized as a basis for modifying or designing other structures for carrying out the same purposes of the present invention. It should also be realized by those skilled in the art that such equivalent constructions do not depart from the spirit and scope of the invention as set forth in the appended claims. The novel features which are believed to be characteristic of the invention, both as to its organization and method of operation, together with further objects and advantages will be better understood from the following description when considered in connection with the accompanying figures. It is to be expressly understood, however, that each of the figures is provided for the purpose of illustration and description only and is not intended as a definition of the limits of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] For a more complete understanding of the present invention, reference is now made to the following descriptions taken in conjunction with the accompanying drawings.

[0021] FIG. 1 shows that loss of integrated gene by homologous recombination generates a high background in suppressor screens.

[0022] FIG. 2 illustrates a kan^r gene adjacent to the protease gene for identifying loss by homologous recombination.

[0023] FIG. 3 illustrates plasmid integrated at the nmt1 locus by homologous recombination.

[0024] FIG. 4 shows that two G418^r strains were isolated and their growth is strongly inhibited when the protease gene is expressed.

[0025] FIG. 5 demonstrates that cells on an inducing plate do not form colonies.

[0026] FIG. 6 illustrates loss of viability after protease expression.

[0027] FIG. 7 indicates that protease activity can be monitored by relocalization of a GFP-Vpr fusion.

[0028] FIG. 8 demonstrates that HIV-1 protease does not affect localization of GFP.

[0029] FIG. 9 shows that protease does not relocalize GFP-Vpr to the cytoplasm.

[0030] FIG. 10 indicates that protease relocalizes GFP in GFP-SL Ma-vpr to cytoplasm.

[0031] FIG. 11 shows that protease relocalizes GFP in GFP-SL p6-Vpr to cytoplasm.

[0032] FIG. 12 demonstrates that protease does not relocalize GFP in GFP-SL A-Vpr to cytoplasm.

[0033] FIG. 13 illustrates that the PrIntA G418^r strain inhibits growth more than the PrIntB strain.

[0034] FIG. 14 shows that both exemplary strains have a single plasmid integrated at the nmt1 locus, although the sequence of the nmt1 promoter is different.

[0035] FIG. 15 demonstrates that the %GFP pattern indicates the amount of protease activity.

[0036] FIG. 16 indicates that indinavir suppresses the effect of protease on growth.

[0037] FIG. 17 shows that indinavir prevents relocalization of GFP in GFP-SL p6-vpr to cytoplasm.

[0038] FIG. 18 illustrates that GFP pattern decreases with increasing indinavir concentration.

[0039] FIG. 19 shows that indinavir allows colony formation.

[0040] FIG. 20 demonstrates that only about 1 in 1000 cells forms a colony on inducing plates.

[0041] FIG. 21 shows that most colonies on the inducing plate have lost the *kan^r* gene.

[0042] FIG. 22 demonstrates that when colonies from the G418 replica are retested on inducing plates, most still have the protease gene.

[0043] FIG. 23 addresses low background in screening for protease suppressors with the PrIndA strain.

[0044] FIG. 24 shows an example of background, including G418^r and strong growth on –T plates.

[0045] FIG. 25 shows that strong growth on –T media is not dependent on plasmid.

[0046] FIG. 26 shows that both background strains with normal growth on inducing plates have a frameshift mutation in the protease gene.

[0047] FIG. 27 illustrates isolation of weak suppressor plasmids.

[0048] FIG. 28 demonstrates that weak suppression depends on plasmid.

[0049] FIG. 29 shows that after recovery in *E. coli*, one exemplary plasmid (pPS3-2) does not re-test as a suppressor although others do.

[0050] FIG. 30 shows that ten of eleven recovered plasmids re-test as weak suppressor by growth.

[0051] FIG. 31 illustrates that ten of eleven recovered plasmids re-test as weak suppressors by increased cell viability.

[0052] FIG. 32 demonstrates that the ten exemplary suppressor plasmids all comprise the *hhp2* gene.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[0053] In keeping with long-standing patent law convention, the words “a” and “an” when used in the present specification in concert with the word comprising, including the

claims, denote “one or more.” Some embodiments of the invention may consist of or consist essentially of one or more elements, method steps, and/or methods of the invention. It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

II. Embodiments of the Present Invention

[0054] HIV/AIDS is one of the most devastating diseases in the world with approx. 40 million people living with HIV in 2005 and approx. 4.0 million new infections each year. In the U.S., HIV is the second leading killer of adults aged 25 to 44 years. The HIV-1 protease is the most characterized and successful drug target for treatment of HIV infection. The protease is produced as a largely inactive precursor in the Gag-Pol polyprotein as the result of a frame shift during translation. The protease eventually cuts at nine sites in this polyprotein to release the proteins necessary for assembly of infectious virus including release of the fully active protease. Since processing by the protease is essential for the formation of infectious viral particles, the protease has been a major target for anti-HIV drugs, and the protease inhibitors (PI) which have been developed are a main stay of effective anti-HIV treatment. Use of a PI by itself can reduce viral load by two to three logs, and in combination with other antivirals can reduce the virus to undetectable levels. One problem with the currently used PIs is side effects such as hyperlipidemia. Another serious problem with long term use of a PI is the occurrence of resistance mutations in the virus. Some of these resistance mutation are specific for the PI used for treatment, but multiple mutations accumulating in the protease during PI treatment in a patient may lead to resistance to most or all of the PI (Palmer, Shafer, and Merigan, 1999; Vickrey et al., 2003). Cross resistance may be higher than expected in patients and can lead to patient failing treatment with any available PI (Palmer, Shafer, and Merigan, 1999). For example, the MDR769 protease, which was isolated from a patient failing therapy, is more than 40 fold resistant to all approved PI except for a 14 fold resistance to amprenavir (Logsdon et al., 2004). The generation of these multidrug resistance proteases during treatment is a major reason that PI eventually loses effectiveness against HIV infections, raising the possibility that HIV multi-drug resistance may ultimately outgrow the number of PIs that are currently available. Therefore, there is an urgent need to develop additional PIs that are active against these resistant proteases.

[0055] Thus, the treatment of HIV-1 infections would benefit from new PI with reduced side effects and that are active against proteases resistant to available PI. A cell based

screening procedure for new PI using fission yeast would have two advantages. First, it has the typical advantages of a cell based assay where toxic compounds and compounds unable to cross the cell membrane are likely to be removed from the screen but compounds active in the cellular environment should test as positive. Second, the screen would find compounds that inhibit the protease regardless of whether they bind at the active site of the enzyme. All FDA-approved PI as of 2005 bind the active site of the protease and their development relied heavily on detailed structural knowledge of the active site. A screening procedure based only on protease activity might find inhibitors targeting other regions and properties of the protease. An example of a new mechanism to inhibit protease, and one which that could be detected only in a cell based assay, is a compound that targets degradation of the protease by the 26S proteasome. Unfolded proteins, resulting from improper folding of newly synthesized proteins or by denaturation of native proteins, are polyubiquitinated to target them for proteolysis by the 26S proteasome (Wolf and Hilt, 2004) . A compound that transiently prevents proper folding of newly synthesized protease might lead to degradation by the 26S proteasome, but such a compound might not be detected in a cell-free screen where the protease is not newly synthesized and proteasomes are not present. In terms of this example, it is important to note that there is a great deal of similarity in the 26S proteasome degradation pathways for unfolded proteins between fission yeast and human cells (Parodi, 1999) and that this similarity holds for many other basic cellular processes (Zhao and Elder, 2000; Zhao and Lieberman, 1995). Thus, for compounds that require a cellular process to be active, fission yeast is a good system in which to develop a cell based screen for compounds that will be active in human cells.

[0056] Thus, the inventors have developed a system in fission yeast where induced expression of the HIV-1 protease gene causes cell death. In addition to effects on cell growth and viability, the present invention provides an assay for protease activity in fission yeast cells where relocalization of green fluorescence of GFP from the nuclear envelope to throughout the cell indicates protease activity. The PI indinavir inhibits the protease in the cell, as indicated by its effect on growth and the GFP relocalization assay. There are three readouts from this fission yeast system that can be used to develop a HTS system. In addition, incorporation of mutant, resistant protease genes into the developed HTS system allows screening for compounds with broad spectrum activity against wild-type and mutant proteases. These compounds identified in such screens would be used to develop PI against these resistant proteases found in patients failing current treatment regimens.

[0057] At least some embodiments of the present invention concern the isolation of anti-protease inhibitors, including, for example, those directed to HIV-1 protease, HIV-2 protease, or both. In certain aspects of the invention, a fission yeast system allows for rapid and high throughput screening of anti-protease agents in live cells, for example. Because of the intrinsic difference between this newly developed live cell system and the traditional structure-based design, use of this innovative system to screen new anti-PR drugs provides at least one additional regimen for anti-HIV therapy.

III. HIV-1 Protease

[0058] A protease is an enzyme that cleaves proteins to their component peptides. The proteins that comprise the human immunodeficiency virus (HIV) are generated as long "polyproteins" that are cleaved to yield the active protein components of the mature virus. The HIV-1 protease is an aspartic protease that cleaves the nascent polyproteins during viral replication.

[0059] HIV-1 protease (PR) facilitates a maturation process that occurs as the virion buds from the host cell by hydrolyzing viral polyproteins into functional protein products that are essential for viral assembly and subsequent activity. PR is a homodimer, and each monomer comprises 99 amino acids, is identical in conformation, and comprises an N-terminal Pro and C-terminal Phe. The position of each monomer in the active protease forms an axis of symmetry. The secondary structure of each monomer includes one α -helix and two antiparallel β sheets.

[0060] In addition to the dimer being stabilized by noncovalent interactions, hydrophobic packing of side chains and interactions involving the catalytic residues, aliphatic residues stabilize each monomer in a hydrophobic core. Each monomer comprises two cysteine residues, although these do not participate in disulfide bonds. The active site forms at the dimer interface and is generated by a cleft between the two domains as part of a four stranded β turn and is positioned in approximately the center of the molecule. The active site is covered by an extended turn, a β hairpin loop, of a beta sheet. This flap, which extends from Met-46 to Lys-55, remains flexible, allows for hinge-like mobility, and facilitates substrate access to the active site by opening and folding the tips into hydrophobic pockets, thereby exerting a central role in PR activity. PR is oblong and relatively flat, with several potential binding pockets present inside the hollow cleft.

[0061] Catalysis of protein chain cleavage by this protease is mediated by twin Asp-25 residues juxtaposed in the active site. These two aspartates are said to form a "catalytic diad". One of these carboxyl groups exhibits an unusually low pKa of 3.3 and the other displays an unusually high pKa of 5.3. This catalytic diad of aspartate residues normally interacts with the amide bond to be cleaved in the polypeptide substrate.

[0062] In a specific embodiment of the invention, the HIV-1 protease refers to human HIV-1 protease, and in further specific embodiments the HIV-1 protease polynucleotide sequence refers to SEQ ID NO:1 and the polypeptide sequence refers to SEQ ID NO:2. In a specific embodiment of the invention, the HIV-2 protease refers to human HIV-2 protease, and in further specific but exemplary embodiments a HIV-2 protease polypeptide sequence refers to SEQ ID NO:5 (GenBank® Accession No. 2MIPC, gi:443404) and an exemplary HIV-2 protease polynucleotide sequence refers to SEQ ID NO:6. In additional embodiments, the National Center for Biotechnology Information's database GenBank® Accession No. S42993, gi:254697; SEQ ID NO:7, which encodes HIV1 viral infectivity factor (vif) may be employed. In alternative embodiments, the HIV-1 protease or HIV-2 protease may have one or more variations in sequence from the respective provided sequences herein, so long as when the protease is overexpressed in a yeast cell, the cell dies. In particular, there may be a sequence employed in the invention that is at least 70%, 75%, 80%, 85%, 90%, 95%, 97%, 99%, or 100% identical to a sequence provided herein.

[0063] Currently known inhibitors of HIV-1 protease include, for example, Invirase® (saquinavir mesylate (The chemical name for saquinavir mesylate is N-tert-butyl-decahydro-2-[2(R)-hydroxy-4-phenyl-3(S)-[[N-(2-quinolylcarbonyl)-L-asparaginy]amino]butyl]-(4aS, 8aS)-isoquinoline-3(S)-carboxamide methanesulfonate with a molecular formula $C_{38}H_{50}N_6O_5 \cdot CH_4O_3S$ and a molecular weight of 766.96. The molecular weight of the free base is 670.86.)); those inhibitors described in U.S. Patent No. 6,043,357; Statutory Invention Registration H1,649; Ritonavir (Norvir®); amprenavir, atazanavir, fosamprenavir, indinavir, lopinavir, tripranavir and nelfinavir (Viracept®), for example. Inhibitors identified by the present invention may be utilized instead of or in addition to one or more currently known inhibitors of HIV-1 protease.

IV. Candidate HIV-1 Protease Inhibitors

[0064] In particular aspects of the invention, an inhibitor of HIV-1 protease is identified in the screening methods of the invention that are directed to identifying suppressors of

HIV-1 protease. The inhibitor(s) may be of any suitable kind, so long as it is able to suppress death of a yeast cell following overexpression of HIV-1 protease, in certain embodiments. In alternative embodiments, a weak inhibitor is identified, such as by identifying a slow-growing colony upon at least one selection means.

[0065] In specific embodiments of the invention, the HIV-1 protease inhibitor is comprised of one or more proteins, polypeptides, peptides, antibodies, polynucleotides, including DNA and/or RNA, small molecules, synthetic compounds, natural compounds, and so forth. The inhibitor may be identified among a library of other similar types of molecules. For example, there may be employed a library of candidate modulators, including libraries from a variety of organisms, such as *S. pombe* and *S. cerevisiae*; human, mouse, rat, algae, fungus, *Drosophila*; *C. elegans*; *Arabidopsis*; and *Fugu rubripes*, for example. Alternatively, there may be used a library of small molecules, for example. One example of a library is a collection of molecules from one or more plants; an exemplary group of plants from which a library may be utilized includes Chinese herbs. The library may include interference RNA, including RNAi, siRNA, and shRNA, for example. In certain aspects, the small molecule library may be selected based on known structures of protease inhibitors, including known HIV-1 and/or HIV-2 inhibitors.

V. Yeast

[0066] Yeast are unicellular fungi whose mechanisms of cell-cycle control are remarkably similar to that of humans. The precise classification is a field that uses the characteristics of the cell, ascospore and colony. Physiological characteristics are also used to identify species. One of the more well known characteristics is the ability to ferment sugars for the production of ethanol. Budding yeasts are true fungi of the phylum *Ascomycetes*, class *Hemiascomycetes*. The true yeasts are separated into one main order *Saccharomycetales*. Yeasts are characterized by a wide dispersion of natural habitats, and are common on plant leaves and flowers, soil and salt water. Yeasts are also found on the skin surfaces and in the intestinal tracts of warm-blooded animals, where they may live symbiotically or as parasites.

[0067] Yeasts multiply as single cells that divide by budding (*e.g.*, *Saccharomyces*) or direct division (fission, *e.g.* *Schizosaccharomyces*), or they may grow as simple irregular filaments (mycelium). In sexual reproduction most yeasts form asci, which contain up to eight haploid ascospores. These ascospores may fuse with adjoining nuclei and multiply through vegetative division or, as with certain yeasts, fuse with other ascospores.

[0068] Yeast cells that may be used in accordance with the present invention include, but are not limited to, *Saccharomyces* species (e.g., *S. cerevisiae*; *S. carlsbergensis*), *Schizosaccharomyces* species (e.g. *S. pombe*), *Pichia* species (e.g. *P. pastoris*), *Hansenula* species (e.g., *H. polymorpha*), *Kluyveromyces* species (e.g., *K. lactic*), *Yarrowia* species (e.g. *Y. lipolytica*).

[0069] In particular aspects of the invention, the yeast cell that is employed is *Schizosaccharomyces pombe*. The power of yeast genetics is partially due to the ability to quickly map a phenotype producing gene to a region of the *S. pombe* genome. *S. pombe* has been a model system for much of molecular genetic research.

A. Yeast Cultures

[0070] Some yeast varieties reproduce almost as rapidly as bacteria and have a genome size less than 1% that of a mammal. They are amenable to rapid molecular genetic manipulation, whereby genes can be deleted, replaced, or altered. They also have the unusual ability to proliferate in a haploid state, in which only a single copy of each gene is present in the cell. This makes it easy to isolate and study mutations that inactivate a gene as one avoids the complication of having a second copy of the gene in the cell.

[0071] The process of culturing yeast strains involves isolation of a single yeast cell, maintenance of yeast cultures, and the propagation of the yeast until an amount sufficient for pitching is obtained. Pure yeast cultures are obtained from a number of sources such as commercial distributors or culture collections. Various procedures are used to collect pure cultures, including culturing from a single colony, a single cell, or a mixture of isolated cells and colonies.

[0072] The objective of propagation is to produce large quantities of yeast with known characteristics in as short a time as possible. One method is a batch system of propagation, starting with a few milliliters of stock culture and scaling up until a desired quantity of yeast has been realized. Scale-up introduces actively growing cells to a fresh supply of nutrients in order to produce a crop of yeast in the optimum physiological state.

B. Yeast Promoters

[0073] In certain aspects, a regulatory region, which may be referred to as a promoter, is employed to drive expression of HIV protease such that the cell in which it resides dies. The promoter may be present on a plasmid (or other vector) operably linked with the

protease polynucleotide, or the promoter may be present within the genome of the yeast. The promoter may be considered overexpressing, inducible, or constitutive, for example.

[0074] Thus, the expression construct comprising HIV-1 protease comprises a regulatory sequence, wherein the regulatory sequence directs expression of HIV-1 protease to the extent that the cell dies upon its expression. For example, the regulatory sequence may be considered one that overexpresses HIV-1 protease such that it kills the yeast cell. In other cases, the regulatory sequence may be inducible, such as induced to direct expression of HIV-1 protease upon exposure to particular media, for example. One such promoter is the Gal 1,10 promoter, which is inducible by galactose. It is frequently valuable to be able to turn expression of the gene on and off so one can follow the time-dependent effects of expression, for example.

[0075] In certain aspects, the yeast cell is a fission yeast cell, and exemplary promoters for fission yeast include *adh1*⁺ (constitutive high expression), *fbp1*⁺ (carbon source responsive), a tetracycline-repressible system based on the CaMV promoter, and the *nmt1*⁺ (no message in thiamine) promoter, which is currently the most frequently used.

[0076] There are three versions of *nmt1*⁺ promoter: the full strength promoter, and two attenuated versions that have reduced activity both in repressed and induced conditions. Several different polylinkers are available in the REP/RIP series of *nmt* vectors. The concentration of thiamine can be adjusted for partial activation. Full induction: no thiamine. Full repression: 20 μ M thiamine (5 μ g/ml). Partial induction (described in this reference): 0.05 μ M thiamine (0.016 μ g/ml).

[0077] The *nmt1* promoter does not switch off completely, and the ability to construct a "shut-off" plasmid depends very much on the protein being expressed and the sensitivity of the cell to dosage of that particular protein. Many genes expressed under *nmt1* control are able to complement even in the presence of thiamine in the weakest promoter, but there are also numerous examples of genes that can be successfully shut off to generate a null phenotype. Thus, the utility of this promoter for plasmid shut-off experiments must be determined empirically for each gene.

[0078] Other useful exemplary yeast promoters for the conditional expression of HIV-1 protease include those directing expression of *nmt1*, *fbp1*⁺, *inv1*⁺ and *ctr4*⁺, and one may consider these promoters useful for *S. pombe*, for example. Other promoters, which may be considered useful for *S. cerevisiae*, for example, include metallothionein, 3-phosphoglycerate

kinase or other glycolytic enzymes I such as enolase or glyceraldehyde-3-phosphate dehydrogenase, enzymes responsible for maltose and galactose utilization, and others. Other examples of a strong yeast promoter are the alcohol dehydrogenase, lactase and triosephosphate I isomerase promoters.

[0079] The Gal 1 gene and Gal 10 gene are adjacent and transcribed in opposite directions from the same promoter region. The regulatory region containing the UAS sequences can be cut out on a DdeI Sau3A fragment and placed upstream of any other gene to confer galactose inducible expression and glucose repression. The PGK, GPD and ADH1 promoters are high expression constitutive promoters (PGK = phosphoglycerate kinase, GPD = glyceraldehyde 3 phosphate dehydrogenase, ADH1 = alcohol dehydrogenase). The ADH2 promoter is glucose repressible and it is strongly transcribed on non-fermentable carbon sources (similar to GAL 1 or 10) except not inducible by galactose. The CUP1 promoter is the metallothionein gene promoter. It is activated by copper or silver ions added to the medium. The CUP1 gene is one of a few yeast genes that is present in yeast in more than one copy in the genome. Depending on the strain, there can be up to eight copies of this gene. The PHO5 promoter is a secreted gene coding for an acid phosphatase. It is induced by low or no phosphate in the medium. The phosphatase is secreted in the chance it will be able to free up some phosphate from the surroundings. When phosphate is present, no PHO5 message can be found. When it is absent, it is turned on strongly.

C. Yeast Transformation Protocols

[0080] A variety of approaches are available for transforming yeast cells and include electroporation, lithium acetate and protoplasting. In one embodiment, a molecule may be delivered into a yeast cell simply by growing the yeast colony in media comprising the molecule. In certain embodiments of the present invention, a molecule is introduced into an organelle, a cell, a tissue or an organism *via* electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge. In some variants of this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells (U.S. Patent No. 5,384,253, incorporated herein by reference). Zymolase may be used at least for *S. pombe*. Alternatively, recipient cells can be made more susceptible to transformation by mechanical wounding.

[0081] Protoplast fusion has been used to overcome sexual barriers that prevent genetically unrelated strains from mating (Svoboda, 1976), thus facilitating the total or partial

exchange of genetic components (Provost *et al.*, 1978; Wilson *et al.*, 1982; Perez *et al.*, 1984; Spencer *et al.*, 1985; Pina *et al.*, 1986; Skala *et al.*, 1988; Janderova *et al.*, 1990; Gupthar, 1992; Moluar and Sipiczki, 1993). The process relies on cell wall digestion followed by fusion with, *e.g.*, polyethylene glycol (Kao and Michayluk, 1974) and the protoplast adhesion promoter, Ca^{2+} , have been exploited in yeast fusion experiments (van Solingen and van der Plaat, 1977; Svoboda, 1978; Wilson *et al.*, 1982; Pina *et al.*, 1986). Other workers report "an enhancement of the protoplast fusion rate" using electro-fusion techniques instead of polyethylene glycol (Weber *et al.*, 1981; Halfmann *et al.*, 1982). The action of polyethylene glycol is not specific. It catalyses the aggregation of protoplasts between the same or different species.

[0082] The fusion process may be summarized as follows: (i) random aggregation of protoplasts into clumps of various sizes (Anne and Peberdy, 1975; Sarachek and Rhoads, 1981); (ii) conversion of the aggregates into syncytia ("chimaeric protoplast fusion product") by dissolution of membranes and merging of cytoplasmic contents (Ahkong *et al.*, 1975a; Gumpert, 1980; Svoboda, 1981; Sarachek and Rhoads, 1981; Klinner and Bottcher, 1984); (iii) membrane organization (Ahkong *et al.*, 1975a; Gumpert, 1980) and fusion of nuclei within heterokaryons (Sarachek and Rhoads, 1981; Klinner and Bottcher, 1984).

[0083] Another approach uses electroporation. Cells are first grown to a density of about $1 \times 10^7/\text{ml}$ ($\text{OD}_{595} = 0.5$) in minimal medium (transformation frequency is not harmed by growth until early stationary phase ($\text{OD}_{595} = 1.5$)). Cells are harvested by spinning at 3000 rpm for 5 minutes at 20°C , followed by washing once in ice-cold water and washing a second time in ice-cold 1M sorbitol. It has been reported (Suga and Hatakeyama, 2001), that 15 min incubation of these cells in the presence of DTT at 25 mM increases electrocompetence. The final resuspension is in ice-cold 1M sorbitol at a density of $1 - 5 \times 10^9/\text{ml}$. Forty μl of the cell I suspension are added to chilled tubes containing the DNA for transformation (100 ng) and incubated on ice for 5 minutes. The electroporator may be set as follows: (a) 1.5kV, 200 ohms, 25 μF (Biorad); (b) 1.5 kV, 132 ohms, 40 μF (Jensen/Flowgen). Cells and DNA are transferred to a pre-chilled cuvette and pulsed; 0.9 ml of ice-cold 1M sorbitol is then immediately added to the cuvette; the cell suspension is then returned to the tube and placed on ice while other electroporations are carried out. Cells are plated as soon as possible onto minimal selective medium. Transformants should appear in 4-6 days at 32°C

[0084] The following lithium acetate protocol is derived from Okazaki *et al.* (1990), High frequency transformation method and library transducing vectors for cloning

mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe*. Cells are grown in a 150 ml culture in minimal medium to a density of $0.5 - 1 \times 10^7$ cells/ml ($OD_{595} = 0.2-0.5$). Media with low glucose, or MB media (see Okazaki *et al.*), in which the cells are less happy, may increase transformation efficiency. Cells are harvested at 3000 rpm for 5 minutes at room temperature, then washed in 40 ml of sterile water and spun down as before. The cells are resuspend at 1×10^9 cells/ml in 0.1 M lithium acetate (adjusted to pH 4.9 with acetic acid) and dispensed in 100 μ l aliquots into eppendorf tubes. Incubation is at 30°C (25°C for ts mutants) for 60 - 120 min. Cells will sediment at this stage. One μ g of plasmid DNA in 15 μ l TE (pH 7.5) is added to each tube and mix by gentle vortexing, completely resuspending cells sedimented during the incubation. The tubes should not be allowed to cool down at this stage. 290 μ l of 50 % (w/v) PEG 4000 pre-warmed at 30°C (25°C for ts mutants) is added. Next, mix by gentle vortexing and incubate at 30°C (25°C for ts mutants) for 60 minutes. The tubes are heat shocked at 43°C for 15 minutes, followed by cooling to room temperature for 10 minutes. The tubes are then centrifuged at 5000 rpm for 2 minutes in an eppendorf centrifuge. The supernatant is carefully removed by aspiration. Cells are re-suspend in 1 ml of 1/2 YE broth by pipetting up and down with a pipetman P1000, transferred to a 50 ml flask and diluted with 9 ml of 1/2 YE. The cells are incubated with shaking at 32°C (25°C for ts mutants) for 60 minutes or longer. Aliquots of less than 0.3 ml are plated onto minimal plates. If necessary, cells are centrifuged at this stage and resuspended in 1 ml of media to spread more cells on a plate.

D. Yeast Codon Bias

[0085] To obtain optimal expression of a heterologous peptidase in yeast cells, nucleic acids encoding peptidases are designed and synthesized according to yeast codon preference. The following table provides exemplary yeast codon preferences for *S. pombe*, for example.

[0086] Table 1: *Schizosaccharomyces pombe* Codon Preferences

Gly	GGT, GGA
Glu	GAA
Asp	GAT
Val	GTT

Ala	GCT
Lys	AAA
Asn	AAT
Ile	ATT
Thr	ACT
Cys	TGT
End	TAA
Tyr	TAT
Leu	TTG, TTA, CTT
Phe	TTT
Ser	TCT
Arg	CGT
Gln	CAA
His	CAT
Pro	CCT

E. Yeast Markers

[0087] In certain aspects of the invention, a marker is utilized to monitor the presence of particular polynucleotides. The marker may be considered a nutritional marker or may be considered an antibiotic resistance marker, for example. One or more markers may be utilized. Although any nutritional marker that allows detection of the presence of the marked polynucleotide may be employed, in certain aspects the nutritional marker includes *ade1*, *ade6*, *arg3*, *CAN1*, *his3*, *his7*, *leu1*, *leu2*, *sup3-5*, *ura4*, and *ura3*. Also, although any antibiotic resistance marker that allows detection of the presence of the marked polynucleotide may be employed, in certain aspects the antibiotic for which resistance is tested includes kanamycin, G418, and hygromycin.

F. Yeast Expression Vectors

[0088] In certain embodiments, one or more compositions associated with the present invention are comprised in an expression vector. Although any suitable vector may be utilized, in specific embodiments, one or more fission yeast vectors are utilized, such as follows in Table 2, which may be found at least in part at a World Wide Web website of Susan Forsberg at the University of Southern California.

[0089] Table 2: Exemplary General Purpose Fission Yeast Vectors

plasmid name	base plasmid	yeast markers	yeast origin	other features and comments
pAL19	pUC	LEU2	ars1	blue/white
paR3		arg3+	ars1	
pBG1	pUC	his3+	ars1	blue/white
pDBlet	bluescript	<i>ura4+</i>	2x ars3002	dual ars for increased stability
pDB248X	pBR	LEU2	2 micron	
pEA500	pSP2	his7+	ars1	
pFL20	pBR322	URA3	ars1 stb	
pIRT2 pIRT2U	pUC	LEU2 <i>ura4+</i>	ars1	
PIRT2-CAN1	pUC	LEU2 CAN1	ars1	CAN1 for counter-selection in can 1-1 strains
PJK148 pJK210	bluescript	leu1+ <i>ura4+</i>	--	blue/white integrating
pON163		<i>ura4+</i>	ars1	positive selection for inserts with kanamycin

pNPT/ADE1-3	pART	adh- neoR ade1+	ars1	can be selected for with G418
pSP1 pSP2		LEU2 URA3	ars1	Complements leu1 strains complements <i>ura4</i> strains
pSP3		HIS3		complements his5 strains
pSP4		LYS2	ars1	complements lys1 strains
pUR18 pUR19	pUC	<i>ura4+</i>	ars1	blue/white
pZA57	pEA500	his7+ ade1+	ars1	pZA58 has ade1+ in opposition orientation
pWH5		LEU2	2μ	First generation vector used in early cloning expts. Now replaced by plasmids such as pAL19, <i>etc.</i>

Table 3: Fission Yeast Expression Vectors

name	base plasmid	yeast markers	yeast origin	promoter
pART1	pIRT/pUC	LEU2	ars	adh promoter
pCHY21		URA3	ars1	fbp promoter
pEVP11		LEU2	2 μ	adh promoter
REP1, REP3, REP4	pUC	LEU2 <i>ura4+</i>	ars1	nmt full strength
REP41,	REP1,2	LEU2	ars1	nmt med strength (nmt*)

REP42		<i>ura4+</i>		
REP81, REP82	REP1,2	LEU2 <i>ura4+</i>	ars1	nmt low strength (nmt**)
RIP	derived from above: integrating vectors lacking ars1. RIP-s vectors contain sup3-5 on PstI fragment.			
REP3X REP4X REP41X REP81X REP42X REP82X RIP3Xs RIP4Xs	The REP-X derivatives are derived from the REP/RIP series above. The original REP/RIP vectors contain an ATG at the 5' end of the polylinker; the X-series removes it and adds a XhoI site. Note that you must supply an ATG on your clone if using the X-series.			
pYZ1N pYZ41N pYZ81N	Derived from the REP series, with a lacZ alpha peptide in the polylinker to allow blue/white selection for inserts.			
pSLF101 pSLF102	pUC	LEU2 <i>ura4+</i>	ars1	CaMV with tet operator repressed by adh-tet repressor (from integrated pSLF104)
pSLF104	pUC	sup3-5	--	
pSM1/2		LEU2	2 micron	SV40 promoter
pTLM2/pAL7 (pAU5)		pSV40- neoR	none	hCMV promoter; titrating G418 allows increase in copy number
p2UG		URA3	2 micron	GRE elements regulated

pART1/N795		LEU2	ars1	by adh-glucocorticoid receptor (pART)
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VI. Assays

[0090] As discussed above, the yeast cell system of the present invention is designed such that there is identification of a suppressor that rescues a yeast cell from cell death caused by overexpression of HIV-1 protease and/or identification of a suppressor that prevents enzymatic activity of the protease, such as cleavage of a protease substrate.

[0091] In one embodiment of assay development, there is optimization and validation of one or more fission yeast-based assays for an exemplary 96-well microtiter plate format. Specifically, the following will occur:

[0092] 1) convert an agar-based plating assay for measuring growth inhibition induced by HIV-1 protease in the RE294 fission yeast strain, into an absorbance assay;

[0093] 2) adapt a yeast live/dead fluorescent readout for determination of protease-induced cell death;

[0094] 3) convert a sub-cellular relocalization assay followed by GFP fluorescence into a high-density fluorescent assay to monitor the activity of HIV-1 protease; this assay is used as a counter screen test.

[0095] In an embodiment for configuration of assays for HTS, phase II optimization of the developed assays are carried out to further configure assays for HTS. Specifically, one can:

[0096] 1) configure all of the HIV-1 protease assays into the HTS formats, further miniaturized if possible;

[0097] 2) interface the SAMI NT Software Integration of Laboratory Automation Systems with the Core HTS system for control of all assay performance including liquid handling and incubation;

[0098] 3) establish all reaction criteria based on quality control parameters including assay reproducibility, CV, signal/background ratio and Z' factor analysis;

[0099] 4) carry out test runs by using a pharmacologically active LOPAC1280 compound library (Sigma-RBI).

[0100] After the proposed adaptation and validation, these fission yeast cell-based assays are ready for HTS screening for new HIV-1 protease inhibitors by participating in the Molecular Libraries Screening Centers Network (MLSCN), for example.

[0101] Cleavage by HIV protease may be tested in any suitable manner, although in particular embodiments it is tested for by assaying for cleavage of a particular HIV protease site. Any HIV protease site may be employed, although in particular aspects the site comprises DSQNYPIVQ (SEQ ID NO:3); DSFNFPQIT (SEQ ID NO:4); VSQNYPIVQN (SEQ ID NO:8); KARVLAEAMS (SEQ ID NO:9); SATIMMQRGN (SEQ ID NO:10); RPGNFLQSRP (SEQ ID NO:11); VSPNFPQITL (SEQ ID NO:12); CTLNFPISPI (SEQ ID NO:13); GAETFYVDG (SEQ ID NO:14); or IRKVLFLDGI (SEQ ID NO:15), for example (see Wlodawer and Gustchina, 2000).

[0102] Expression of the heterologous HIV-1 protease results in death of the yeast host cell, but inclusion of an inhibitor in the presence of the HIV-1 protease, under conditions supporting protease expression, aborts the death caused by the protease and permits proliferation of the yeast cells. One can introduce a large number of biological peptides, proteins, small molecules, or intracellular recombinant antibodies, for example, in yeast cells bearing the heterologous protease to directly and rapidly select/identify specific inhibitors that permit yeast cell growth in the presence of the protease and/or that prevent cleavage of a protease substrate.

A. Genetic Selection

[0103] In genetic selection, a DNA library encoding potential polypeptide inhibitors can be transformed in yeast cells with high frequency (up to 10^7 transformants/microgram plasmid DNA). Transformants are plated on agar plates containing, for example, an inducer of the protease gene expression, and an amino acid drop-out for the selection of plasmid marker. Most yeast transformants would not be able to grow on the plates since the protease is expressed, and those not transformed will additionally not grow because of the absence of the plasmid. However, the presence of a plasmid-borne inhibitor in a yeast transformant will lead at least to cell growth and formation of a colony. The plasmid DNA can be recovered using standard DNA purification procedure, and the DNA sequence of the inhibitor can be determined through DNA sequencing, for example.

[0104] The source of the candidate inhibitors includes a nucleic acid library, including a genomic or cDNA library, a protein library, a peptide library, an RNA library, an

antibody library, a small organic molecule library, and may be of any suitable kind so long as they are transformable into the yeast cell. The genomic libraries may be derived from yeast, including *S. pombe* and *S. cerevisiae*; human, mouse, rat, algae, fungus, *Drosophila*; *C. elegans*; *Arabidopsis*; and *Fugu rubripes*, for example.

B. High-Throughput Screen

[0105] Small molecule peptide and chemical inhibitors can be identified by methods of the invention. Yeast cells are diluted and distributed equally in each well in the presence of the appropriate yeast growth media. Compounds are distributed to each well and yeast cell growth is monitored by visual inspection or measured with a multi-well plate reader (at A_{600}), for example. The presence of an inhibitor will lead to yeast cell growth and increased turbidity in a well. This HTS assay is a standard practice and has been successfully employed in the identification of small molecule inhibitors of process distinct from the present invention (see Hughes, 2002). To overcome the potentially limiting factor of cell penetration, one can enhance cell permeability of the yeast cells by treating with specific chemicals such as polymyxin B (Boguslaski, 1985), or using yeast strain that carries a cell wall mutation (Brendel, 1976). Also contemplated are yeast cells that are impaired in multidrug efflux (Wolfger *et al.*, 2001).

VII. Combination Treatments

[0106] Inhibitors of HIV-1 protease identified by the present invention may be administered to an individual in need thereof, such as an individual infected with HIV, an individual with AIDS, or an individual susceptible to being infected with HIV or develop AIDS. In order to increase the effectiveness of the therapy provided by the present invention, it may be desirable to combine these compositions with other agents effective in the treatment of HIV/AIDS, although in alternative embodiments the inhibitors of the invention are administered without combination with another treatment for HIV or AIDS. More generally, these other compositions would be provided in a combined amount effective to kill the HIV virus and/or cells comprising same, for example. In specific embodiments, the combination therapy with the inventive compositions will comprise a cocktail of therapeutic agents, including a combination itself of different kinds of HIV therapeutics. For example, there may be used one or more protease inhibitors combined with one or more reverse transcriptase inhibitors.

[0107] This process may involve contacting cells of the individual with the inventive composition and the additional agent(s) or multiple factor(s) at the same time. This

may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two or more distinct compositions or formulations, at the same time, wherein one composition includes the inventive composition and the other includes the second agent(s). The combination may provide additive or synergistic effects between the two therapies, for example.

[0108] The therapy of the invention may precede or follow the other agent treatment by intervals ranging from minutes to weeks, for example. In embodiments where the other agent and composition of the invention are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and composition of the invention would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one may contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several d (2, 3, 4, 5, 6 or 7) to several wk (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

[0109] Various combinations may be employed, such as wherein the composition of the invention is "A" and the additional agent is "B":

[0110] A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B

[0111] B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A

[0112] B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

[0113] Administration of the therapeutic inhibitor compositions of the present invention to a patient may follow general protocols for the administration of other HIV/AIDS therapies. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies may be applied in combination with the described inhibitor therapy.

[0114] Additional HIV/AIDS therapies that may be employed in conjunction with the therapy of the present invention includes one or more of at least the following: nonnucleoside reverse transcriptase inhibitors, such as delavirdine, efavirnz, and nevirapine; nucleoside reverse transcriptase inhibitors, such as abacavir, lamivudine, zidovudine, stavudine, tenofovir DF, zalcitabine, and zidovudine; protease inhibitors, such as amprenavir, atazanavir,

fosamprenavir, indinavir, lopinavir, ritonavir, nelfinavir, saquinavir, and tripranavir; and/or fusion inhibitors, such as enfuvirtide, for example.

VIII. Pharmaceutical Preparations

[0115] Pharmaceutical compositions of the present invention comprise an effective amount of one or more HIV-1 and/or HIV-2 protease inhibitors identified by screening methods of the invention and, optionally, an additional agent, dissolved or dispersed in a pharmaceutically acceptable carrier. In a specific embodiment of the invention, the pharmaceutical composition comprises hhp2 polynucleotide or polypeptide, for example. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of an pharmaceutical composition that contains at least one HIV-1 protease inhibitor or additional active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference. Moreover, for animal (*e.g.*, human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

[0116] As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (*e.g.*, antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289-1329, incorporated herein by reference). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the pharmaceutical compositions is contemplated.

[0117] The HIV-1 protease inhibitor may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. The present invention can be administered intravenously, intradermally, transdermally, intrathecally, intraarterially, intraperitoneally, intranasally, intravaginally, intrarectally, topically, intramuscularly, subcutaneously, mucosally, orally, topically, locally, inhalation (*e.g.*, aerosol inhalation),

injection, infusion, continuous infusion, localized perfusion bathing target cells directly, *via* a catheter, *via* a lavage, in cremes, in lipid compositions (*e.g.*, liposomes), or by other method or any combination of the forgoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference).

[0118] The HIV-1 protease inhibitor may be formulated into a composition in a free base, neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts, *e.g.*, those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as formulated for parenteral administrations such as injectable solutions, or aerosols for delivery to the lungs, or formulated for alimentary administrations such as drug release capsules and the like.

[0119] Further in accordance with the present invention, the composition of the present invention suitable for administration is provided in a pharmaceutically acceptable carrier with or without an inert diluent. The carrier should be assimilable and includes liquid, semi-solid, *i.e.*, pastes, or solid carriers. Except insofar as any conventional media, agent, diluent or carrier is detrimental to the recipient or to the therapeutic effectiveness of the composition contained therein, its use in administrable composition for use in practicing the methods of the present invention is appropriate. Examples of carriers or diluents include fats, oils, water, saline solutions, lipids, liposomes, resins, binders, fillers and the like, or combinations thereof. The composition may also comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (*e.g.*, methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

[0120] In accordance with the present invention, the composition is combined with the carrier in any convenient and practical manner, i.e., by solution, suspension, emulsification, admixture, encapsulation, absorption and the like. Such procedures are routine for those skilled in the art.

[0121] In a specific embodiment of the present invention, the composition is combined or mixed thoroughly with a semi-solid or solid carrier. The mixing can be carried out in any convenient manner such as grinding. Stabilizing agents can be also added in the mixing process in order to protect the composition from loss of therapeutic activity, i.e., denaturation in the stomach. Examples of stabilizers for use in the composition include buffers, amino acids such as glycine and lysine, carbohydrates such as dextrose, mannose, galactose, fructose, lactose, sucrose, maltose, sorbitol, mannitol, *etc.*

[0122] In further embodiments, the present invention may concern the use of a pharmaceutical lipid vehicle compositions that include a HIV-1 protease inhibitor, one or more lipids, and an aqueous solvent. As used herein, the term “lipid” will be defined to include any of a broad range of substances that is characteristically insoluble in water and extractable with an organic solvent. This broad class of compounds are well known to those of skill in the art, and as the term “lipid” is used herein, it is not limited to any particular structure. Examples include compounds which contain long-chain aliphatic hydrocarbons and their derivatives. A lipid may be naturally occurring or synthetic (i.e., designed or produced by man). However, a lipid is usually a biological substance. Biological lipids are well known in the art, and include for example, neutral fats, phospholipids, phosphoglycerides, steroids, terpenes, lysolipids, glycosphingolipids, glycolipids, sulphatides, lipids with ether and ester-linked fatty acids and polymerizable lipids, and combinations thereof. Of course, compounds other than those specifically described herein that are understood by one of skill in the art as lipids are also encompassed by the compositions and methods of the present invention.

[0123] One of ordinary skill in the art would be familiar with the range of techniques that can be employed for dispersing a composition in a lipid vehicle. For example, the HIV-1 protease inhibitor may be dispersed in a solution containing a lipid, dissolved with a lipid, emulsified with a lipid, mixed with a lipid, combined with a lipid, covalently bonded to a lipid, contained as a suspension in a lipid, contained or complexed with a micelle or liposome, or otherwise associated with a lipid or lipid structure by any means known to those of ordinary skill in the art. The dispersion may or may not result in the formation of liposomes.

[0124] The actual dosage amount of a composition of the present invention administered to an animal patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. Depending upon the dosage and the route of administration, the number of administrations of a preferred dosage and/or an effective amount may vary according to the response of the subject. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

[0125] In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. In other embodiments, the an active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0126] In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, *etc.*, can be administered, based on the numbers described above.

C. Alimentary Compositions and Formulations

[0127] In preferred embodiments of the present invention, the HIV-1 protease inhibitors are formulated to be administered *via* an alimentary route. Alimentary routes include all possible routes of administration in which the composition is in direct contact with the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered orally, buccally, rectally, or sublingually. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft- shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

[0128] In certain embodiments, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (Mathiowitz *et al.*, 1997; Hwang *et al.*, 1998; U.S. Pat. Nos. 5,641,515; 5,580,579 and 5,792, 451, each specifically incorporated herein by reference in its entirety). The tablets, troches, pills, capsules and the like may also contain the following: a binder, such as, for example, gum tragacanth, acacia, cornstarch, gelatin or combinations thereof; an excipient, such as, for example, dicalcium phosphate, mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate or combinations thereof; a disintegrating agent, such as, for example, corn starch, potato starch, alginic acid or combinations thereof; a lubricant, such as, for example, magnesium stearate; a sweetening agent, such as, for example, sucrose, lactose, saccharin or combinations thereof; a flavoring agent, such as, for example peppermint, oil of wintergreen, cherry flavoring, orange flavoring, *etc.* When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar, or both. When the dosage form is a capsule, it may contain, in addition to materials of the above type, carriers such as a liquid carrier. Gelatin capsules, tablets, or pills may be enterically coated. Enteric coatings prevent denaturation of the composition in the stomach or upper bowel where the pH is acidic. See, *e.g.*, U.S. Pat. No. 5,629,001. Upon reaching the small intestines, the basic pH therein dissolves the coating and permits the composition to be released and absorbed by specialized cells, *e.g.*, epithelial enterocytes and Peyer's patch M cells. A syrup or elixir may contain the active compound sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course,

any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

[0129] For oral administration the compositions of the present invention may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally- administered formulation. For example, a mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an oral solution such as one containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a dentifrice, or added in a therapeutically-effective amount to a composition that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants. Alternatively the compositions may be fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

[0130] Additional formulations which are suitable for other modes of alimentary administration include suppositories. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum. After insertion, suppositories soften, melt or dissolve in the cavity fluids. In general, for suppositories, traditional carriers may include, for example, polyalkylene glycols, triglycerides or combinations thereof. In certain embodiments, suppositories may be formed from mixtures containing, for example, the active ingredient in the range of about 0.5% to about 10%, and preferably about 1% to about 2%.

D. Parenteral Compositions and Formulations

[0131] In further embodiments, HIV-1 protease inhibitor may be administered *via* a parenteral route. As used herein, the term "parenteral" includes routes that bypass the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered for example, but not limited to intravenously, intradermally, intramuscularly, intraarterially, intrathecally, subcutaneous, or intraperitoneally U.S. Pat. Nos. 6,753,514, 6,613,308, 5,466,468, 5,543,158; 5,641,515; and 5,399,363 (each specifically incorporated herein by reference in its entirety)..

[0132] Solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene

glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy injectability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (i.e., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0133] For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

[0134] Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. A powdered composition is combined with a liquid carrier such as, *e.g.*, water or a saline solution, with or without a stabilizing agent.

E. Miscellaneous Pharmaceutical Compositions and Formulations

[0135] In other preferred embodiments of the invention, the active compound HIV-1 protease inhibitor may be formulated for administration *via* various miscellaneous routes, for example, topical (*i.e.*, transdermal) administration, mucosal administration (intranasal, vaginal, *etc.*) and/or inhalation.

[0136] Pharmaceutical compositions for topical administration may include the active compound formulated for a medicated application such as an ointment, paste, cream or powder. Ointments include all oleaginous, adsorption, emulsion and water-solubly based compositions for topical application, while creams and lotions are those compositions that include an emulsion base only. Topically administered medications may contain a penetration enhancer to facilitate adsorption of the active ingredients through the skin. Suitable penetration enhancers include glycerin, alcohols, alkyl methyl sulfoxides, pyrrolidones and luarocapram. Possible bases for compositions for topical application include polyethylene glycol, lanolin, cold cream and petrolatum as well as any other suitable absorption, emulsion or water-soluble ointment base. Topical preparations may also include emulsifiers, gelling agents, and antimicrobial preservatives as necessary to preserve the active ingredient and provide for a homogenous mixture. Transdermal administration of the present invention may also comprise the use of a "patch". For example, the patch may supply one or more active substances at a predetermined rate and in a continuous manner over a fixed period of time.

[0137] In certain embodiments, the pharmaceutical compositions may be delivered by eye drops, intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering compositions directly to the lungs *via* nasal aerosol sprays has been described *e.g.*, in

U.S. Pat. Nos. 5,756,353 and 5,804,212 (each specifically incorporated herein by reference in its entirety). Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, 1998) and lysophosphatidyl-glycerol compounds (U.S. Pat. No. 5,725,871, specifically incorporated herein by reference in its entirety) are also well-known in the pharmaceutical arts. Likewise, transmucosal drug delivery in the form of a polytetrafluoroethylene support matrix is described in U.S. Pat. No. 5,780,045 (specifically incorporated herein by reference in its entirety).

[0138] The term aerosol refers to a colloidal system of finely divided solid or liquid particles dispersed in a liquefied or pressurized gas propellant. The typical aerosol of the present invention for inhalation will consist of a suspension of active ingredients in liquid propellant or a mixture of liquid propellant and a suitable solvent. Suitable propellants include hydrocarbons and hydrocarbon ethers. Suitable containers will vary according to the pressure requirements of the propellant. Administration of the aerosol will vary according to subject's age, weight and the severity and response of the symptoms.

IX. Exemplary Kits of the Invention

[0139] Any of the compositions described herein may be comprised in a kit. In a non-limiting example, an HIV-1 protease inhibitor, such as one identified by the screening methods of the present invention and, optionally, an additional agent, may be comprised in a kit. The kits will thus comprise, in suitable container means, a HIV-1 protease inhibitor and, optionally, an additional agent.

[0140] The kits may comprise a suitably aliquoted HIV-1 protease inhibitor that may be packaged either in aqueous media or in lyophilized form, for example. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there are more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the HIV-1 protease inhibitor and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow molded plastic containers into which the desired vials are retained.

[0141] When the components of the kit are provided in one or more liquid solutions, the liquid solution may be an aqueous solution, with a sterile aqueous solution being particularly preferred. The HIV-1 protease inhibitor compositions may also be formulated into a syringeable composition. In which case, the container means may itself be a syringe, pipette, and/or other such like apparatus, from which the formulation may be applied to an infected area of the body, injected into an animal, and/or even applied to and/or mixed with the other components of the kit.

[0142] However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

[0143] Irrespective of the number and/or type of containers, the kits of the invention may also comprise, and/or be packaged with, an instrument for assisting with the injection/administration and/or placement of the ultimate HIV-1 protease inhibitor composition within the body of an animal. Such an instrument may be a syringe, pipette, forceps, and/or any such medically approved delivery vehicle, for example.

EXAMPLES

[0144] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

EXEMPLARY EMBODIMENTS OF THE INVENTION

[0145] As noted herein, expression of the HIV-1 protease gene from the nmt1 promoter on a plasmid kills fission yeast. This ability to kill fission yeast is exploited as the basis of a screen for suppressors of the protease, in particular aspects of the invention. Although

the killing of yeast by protease expression was first demonstrated on a plasmid, because plasmid transformation is easier than integrating a gene into the chromosome, a plasmid replicating independently of the chromosome is not optimal for suppressor screens, in certain aspects of the invention. Plasmids in fission yeast are considerably less stable than the chromosomes, and integrating a gene into the chromosome takes advantage of the inherent stability of the chromosome. Integration also gives more uniform expression of the gene because plasmid copy number varies from 0 to more than 30 leading to a large variation in expression levels.

[0146] The uniform level of expression and the potential for increased stability are clear advantages in using an integrated gene rather than the gene carried on a plasmid in a screen for suppressors. However, the standard method of integrating a gene into a chromosome in fact creates a source of instability (Keeney and Boeke 1994). With this standard method, when a gene is inserted into the chromosome by homologous recombination, often at the *ura4* locus, a duplication of the *ura4* region flanks the integrated gene (FIG. 1). In a process that reverses the integration event, homologous recombination across the duplication leads to loss of the integrated gene. Thus, when trying to select suppressors of the protease with these integrants made by the standard method, in specific embodiments there will be a high background from the colonies that grow from cells that have lost the protease gene.

[0147] The complication of gene loss is increased by characteristics of the exemplary *nmt1* promoter, which is used to express high levels of the protease gene. The *nmt1*, which stands for no message in thiamine, promoter is controlled by vitamin B1 thiamine. With high levels of thiamine in the media (+T), the *nmt1* promoter is repressed, and when the cell is depleted of thiamine, the promoter is induced. The cell actively concentrates and stores thiamine from the media, and a shift to media without thiamine (–T) does not immediately induce the promoter. Only after the internal stores of thiamine are sufficiently diluted by cell division is the *nmt1* promoter induced to high levels of expression, and at least four cell divisions are necessary to induce the *nmt1* promoter (Tommasino and Maundrell 1991).

[0148] This requirement for cell division means that a single cell plated on inducing media will grow up into a microcolony before the *nmt1* promoter is induced. If just one of the cells in this microcolony loses the gene, that cell will grow up to give a background colony in the suppressor screen. In practice, the rate of gene loss is high enough that in some cases up to 2% of the cells plated on inducing media will give colonies as the result of gene loss. This high level of background colonies can interfere with the identification of suppressors.

[0149] To facilitate identification of authentic suppressors, an exemplary method has been developed that allows the rapid, convenient detection of colonies that are the result of gene loss. The *kan^r* gene is linked to the protease gene in the exemplary construction (FIG. 2) to be integrated into the chromosome. The *kan^r* gene makes fission yeast resistant to the antibiotic G418 on rich YE media. When this construction is integrated at the *nmt1* locus by selecting for transformants resistant to G418 on YE media (FIG. 3), the resulting strains dies on inducing media where the protease gene is expressed. One advantage of using the *kan^r* gene, rather than the more commonly used *ura4⁺* or *leu1⁺* genes (Keeney and Boeke 1994), to make a strain with the integrated protease is that both the *ura4* and *leu1* markers remain available for selection. Most libraries constructed in fission yeast vectors use either *ura4⁺* or *leu1⁺* to select transformants, and both of these types of libraries can be transformed into this strain for screening. However, the primary advantage of the strain made with the *kan^r* gene is that cells that lose the protease gene by homologous recombination will also lose the *kan^r* gene and become sensitive to G418 (FIG. 3). Using the G418 resistance in the screening process described below, the background of colonies on inducing media due to loss of the protease gene can be reduced to less than 1 in 10⁵ cells plated. Reducing the background to these low levels is an essential step in developing a screen to find protease suppressors that are likely to occur at low frequency.

EXAMPLE 2

CHARACTERIZATION OF STRAINS WITH AN INTEGRATED PROTEASE GENE

[0150] Two independent G418-resistant transformants with an integrated protease gene were isolated and characterized. The toxic effects from high level expression of the protease gene was demonstrated in three exemplary ways. First, when incubated in liquid media repressing (+T) or inducing (−T) the *nmt1* promoter, the growth rate in the repressing media was normal, but in the inducing media, growth was strongly inhibited in both strains (FIG. 4). Second, when cells were grown in repressing media and then plated on repressing or inducing media, normal size colonies were seen on the repressing media, but no or very small colonies were seen on the inducing media (FIG. 5). Third, colony-forming ability on repressing media was measured for cells incubated for various times in repressing or inducing liquid media. In repressing media, more than 95% of the cells formed colonies at all time points, but in inducing media the fraction of cells forming colonies decreased rapidly and was less than 1% by 24 hr

after the switch to inducing media (FIG. 6). Because maximum induction of the *nmt1* promoter takes around 14 to 16 hr incubation (Maundrell 1993), cells lose viability a few hours after maximum induction of the protease gene.

[0151] The protease activity of these yeast strains was also demonstrated by an *in vivo* assay for protease activity that was developed based on a fusion between GFP and the HIV-1 protein Vpr (FIG. 7). The green fluorescent protein (GFP) by itself localizes throughout the fission yeast cell (FIG. 8). When GFP is fused to the Vpr protein, the fusion protein localizes according to the signals on the Vpr protein and is found almost exclusively at the nuclear envelope that appears as a small circle of ribbons or dots at the center of the cell with almost no green fluorescence in the rest of the cell (FIG. 9 (Chen, Elder *et al.* 1999)). The assay for protease activity depends on placing a polypeptide linker with a cleavage site for the protease between GFP and vpr (FIG. 7). Without protease activity, the three part fusion protein, GFP, polypeptide linker and Vpr, will be directed to the nuclear envelope by Vpr. With active protease, however, the polypeptide linker will be cleaved and the GFP, which is no longer fused to Vpr, will localize throughout the cell.

[0152] This *in vivo* assay shows that the two strains with the integrated gene have protease activity that cleaves the expected specific substrates. Two control experiments give the expected results. The first control is that the protease does not affect the localization of GFP by itself that localizes throughout the cell with or without the protease (FIG. 8). The second control is that the protease does not affect the localization of the GFP-Vpr to the nuclear envelope when there is no polylinker between GFP and Vpr and therefore no place for cleavage by the protease to release GFP (FIG. 9). However, when a known but exemplary cleavage site for the protease from the junction between the matrix and capsid proteins of HIV-1 (Dunn, Goodenow *et al.* 2002) is placed between GFP and Vpr, the presence of the protease makes a dramatic difference in the localization of the GFP (FIG. 10). Without the protease, the GFP-SL (substrate linker) MA-Vpr localizes to the nuclear envelope, but when the protease gene is expressed, the released GFP localizes through the cell and in most cells there is no longer any obvious labeling of the nuclear envelope. Similarly, when the known cleavage site between p6 and protease of HIV-1 (Dunn, Goodenow *et al.* 2002) is placed between GFP and Vpr, protease expression changes the localization of the GFP from the nuclear envelope to throughout the cell (FIG. 11). A control experiment shows that the substrate linker between GFP and Vpr must contain a protease cleavage site for the relocation to occur. When the substrate linker contains a cleavage site

for the anthrax lethal factor (Cummings, Salowe *et al.* 2002) but no known cleavage site for HIV-1 protease, the GFP-SL A-Vpr fusion protein localizes to the nuclear envelope before and after protease expression (FIG. 12).

[0153] This GFP relocation can be used as a semiquantitative assay for protease activity in the cell and is used to demonstrate a difference between the two strains with an integrated protease gene. When the growth characteristic of the two strains with an integrated protease gene are compared, it is found that the first strain (named PrIntA or RE294) inhibits growth more than the second (named PrIntB or RE295) (FIG. 13). While both strains grow at the normal rate when the *nmt1* promoter is repressed (+T), only PrIntA essentially stops growing in inducing (−T) media while PrIntB grows slowly in inducing media. The stronger effect on growth indicates that PrIntA expresses higher levels of protease, and there is a difference in how PrIntA and PrIntB integrated into the chromosome that might explain the higher expression levels. Both strains have integrated a single copy of the protease gene at the *nmt1* locus, but the exact point of cross over leads to a small sequence difference between the two strain in the *nmt1* promoter (FIG. 14). When expression vectors were developed for the *nmt1* promoter, a NdeI restriction site in the *nmt1* promoter region was removed to allow this site to be used for the insertion of cDNAs (Maundrell 1993). This mutant *nmt1* promoter without a NdeI site is in the vector used to integrate the protease gene, and the chromosome where the integration occurs by homologous recombination has the wild-type sequence with a NdeI site. The exact point of cross over for the homologous recombination determines whether the *nmt1* promoter expressing the protease will have the wild-type or mutant sequence. It was found that the *nmt1* promoter region expressing the protease gene in PrIntA has the wild-type sequence with the NdeI site while PrIntB has the mutant sequence without the NdeI site (FIG. 14). The regulatory sequences of the *nmt1* promoter include the region around the NdeI site (Zurlinden and Schweingruber 1997), and presumably the wild-type sequence in this region in PrIntA leads to higher expression levels and stronger inhibition of growth by the protease.

[0154] The extent of GFP relocation for GFP-SL MA-Vpr also indicates that PrIntA has higher levels of protease activity than PrIntB. When GFP-SL MA-Vpr is present in a cell, the green fluorescence in a cell can be scored as having a distribution pattern typical of GFP, for localization throughout the cell, or as having the Vpr pattern for localization at the nuclear envelope. In this scoring of localization, cells are counted as GFP pattern or Vpr pattern. There are some intermediate cells that have a concentration of green fluorescence at the nuclear

envelope but with some fluorescence throughout the cell, and these intermediate cells are counted as having the Vpr pattern. With this rule for scoring and when there is no protease activity, the GFP is almost exclusively located on the nuclear envelope with 0% having the GFP distribution pattern. When the protease gene in the PrIntA strain is expressed, about 84% of the cells have the GFP localization pattern (FIGS. 10 and 15). However, the PrIntB strain has only 47% of the cells with the GFP pattern. Thus, both the growth rate in inducing media and the fraction of cells with the GFP pattern indicate that PrIntB has less protease activity than PrIntA.

[0155] The protease inhibitor indinavir demonstrates how a suppressor of protease activity affects the PrIntA strain and indicates that this strain can be used to screen for protease suppressors. Indinavir is one of the protease inhibitors, a class of drugs that have been among the most effective in the treatment of HIV-1 infection. These drugs are competitive inhibitors of the protease that act by binding strongly to the active site of the enzyme (Randolph and DeGoey 2004). When PrIntA is treated with indinavir, the effects of inducing the protease gene are reduced. After induction of the protease gene in –T media, indinavir leads to faster growth with concentrations of 12.5 µg/ml and above having the maximum effect where growth through about 25 hr after the shift to –T media approaches that of a culture where the protease gene was not induced (+T) (FIG. 16). The suppression of protease activity is also seen in the relocalization of green fluorescence with the GFP-SL MA-vpr fusion. Most untreated cells have the GFP pattern of localization, but with increasing concentrations of indinavir, cells with the Vpr pattern become more frequent (FIG. 17). Scoring cells for their localization patterns indicates that the GFP pattern decreases with increasing indinavir concentration (FIG. 18), and at the highest concentration of indinavir tested, 200 µg/ml, only a few percent of the cells have the GFP localization pattern.

[0156] Experiments with indinavir directly demonstrate that the PrIntA strains can be used to screen for protease suppressors. The basic idea for the screening procedure is that suppressors will allow PrIntA to form colonies on inducing plates where the toxic effects of protease expression normally prevent formation of normal size colonies. When 500 cells are plated on repressing and inducing plates, most of the cells grow up into colonies by 5 days on the repressing plate, but there no or very small colonies after 5 days incubation on the inducing plate (FIG. 19). (The two larger colonies on the inducing plate in slide 19 result from loss of the protease gene as described below.) Adding indinavir to the inducing media, however, leads to

colony formation, and high concentrations of indinavir lead to colonies close to the size of colonies on the repressing plate.

EXAMPLE 3

TRIALS OF SCREENING PROCEDURES TO MINIMIZE BACKGROUND

[0157] Given this direct demonstration that the PrIndA strain can be used to screen for suppressors, trial experiments were done to establish procedures that minimize the background in the screening process. Some background of cells growing into colonies on inducing plates is expected due to the loss of the protease gene by homologous recombination, but a three step procedure (FIG. 20) nearly eliminates this background from cells that have lost the protease gene. The three step procedure is an exemplary screening process for suppressors, and trials demonstrate that this process eliminates background. The procedure concerns 1) plating under inducing conditions; 2) replicating to G418 plate to test resistance; and 3) checking colonies on the G418 replica for growth under inducing conditions. With these three screening steps in exemplary studies with RE294, the background of colonies resistant to expression of the protease gene is $<1/10^6$.

[0158] In the first step, the PrIndA cells carrying a vector and grown in repressing media are plated on inducing (-T) media. Most of the cells die on the inducing plate due to the expression of the protease gene, but about 1 in a 1000 cells grows into a colony (FIG. 20). It is likely that all or most or all of these colonies are from cells that have lost the protease gene due to homologous recombination across the repeated *nmt1* promoter region, and the loss of the protease gene by this means also leads to loss of the *kan^r* gene (FIG. 3). The *kan^r* gene makes a yeast cell resistant to the antibiotic G418 on rich media, and the presence of the *kan^r* gene is easily determined by replicating the inducing plate to a G418 plate. As a control, the inducing plate is also replicated to a YE plate not containing G418. On this control plate, all of the colonies grow to give the same pattern of colonies as on the original plate. In contrast, few of the colonies from the inducing plate grow on the G418 plate (FIG. 21) indicating that most have lost the *kan^r* gene and that these colonies grew on the inducing media due to loss of the protease gene.

[0159] About 1% of the colonies, however, do grow on the G418 replica (2 are visible in FIG. 21). When examined further, these colonies are not actually resistant to protease expression but appear to arise from a mixture of cells in the original colony on the inducing

plate. When these G418 resistant colonies are rechecked on inducing plates, none of them grew and, by comparison to controls, these strains seem to retain the protease gene and remain just as sensitive to its expression as the starting strain (FIG. 22). The two control strains on the inducing plate are cells from a G418s colony that grow well since they have lost the protease gene (top sector, FIG. 22), and the starting G418r PrIntA strain where most of the cells do not grow and the few large colonies that form are from loss of the protease gene in a small number of cells (adjacent clockwise sector, FIG. 23). When colonies from the G418 replica are retested on this inducing plate, all behave like the original G418r PrIntA strain where most cells do not form normal size colonies (other six sectors, FIG. 22).

[0160] The apparent contradiction between these colonies growing on the original inducing plate but failing to grow when cells from the G418 replica are retested is most likely explained by the colonies being a mixture of G418^r prot⁺ and G418^s prot⁻ cells. During the growth of the colony on the original inducing plate, a cell that loses the protease and kan^r gene will grow rapidly to give a large colony composed mostly of G418^s prot⁻ cells. However, some fraction of cells in this colony remain G418^r prot⁺. When the plate is replicated to a G418 plate, which contains enough thiamine to repress expression of the protease gene, this minority of G418^r prot⁺ cells grows up to give a colony while the G418^s prot⁻ cells are killed by the G418. The cells from the colony on the G418 replica would then not grow on an inducing plate as is seen in FIG. 22.

[0161] In this trial of the screening procedure with PrIndA carrying a vector, more than 1×10^6 cells were plated on inducing media, these plates replicated to G418 and cells from colonies on G418 retested on inducing media. After completing these three screening steps, no G418^r colonies capable of growing on inducing media were isolated (FIG. 23). This result indicates that the background with this three step screening process is low enough that isolation of rare protease suppressors is possible.

EXAMPLE 4

SCREENING OF A FISSION YEAST GENOMIC LIBRARY

[0162] In an exemplary screening of an exemplary plasmid library, there were a few background colonies that passed through the three step the screening process (FIG. 20), but this small background did not seriously interfere with the successful isolation of suppressor plasmids. In addition, adding a fourth step of plasmid loss to the screening procedure was

sufficient in all but one instance to distinguish background strains from authentic plasmid suppressors. The fission yeast genomic library used in this screen was constructed in the pUR18 vector that carries the *ura4* gene (Barbet, Muriel *et al.* 1992). In screening for pUR18 genomic library for protease suppressors, overexpression comes from 5-10 plasmids/cell; G418^r cells were able to grow on –T are plasmid suppressor candidates, and a fourth screening step of testing growth on –T after plasmid loss was useful to eliminate the few false positives that occur, in specific cases.

[0163] Since this is a genomic library, the overexpression of the inserted gene comes from the plasmid copy number which averages around 5 to 10 per cell. PrIntA was transformed with this library and about 40,000 *ura4*⁺ transformants were selected on a repressing plate to prevent expression of the protease gene. The cells were recovered from these transformation plates and plated on inducing media to begin the three step screening process.

[0164] Two strains that appear to strongly suppress the protease were isolated during the screening of these transformants, but when analyzed further, these strains both turned out to have a mutation in the protease gene. These two strains grew well on G418 and on inducing (–T) plates in contrast to the controls with the PrIntA strain transformed with a plasmid from the pUR18 library which grow well on G418 but where most cells do not grow on the inducing plate (FIG. 24). These two strains seemed to be good candidates to carry a protease suppressor on the pUR18 plasmid, but when the plasmid was lost, the strains still grew well on inducing media (FIG. 25). Plasmids from the pUR18 library are unstable in fission yeast, and after growing the strain on media containing uracil that does not select for the plasmid, some cells have lost the plasmid and can no longer grow unless the media is supplemented with uracil. The upper plate in FIG. 25 tests single colonies from the strain grown non-selectively to see whether they have retained the plasmid by determining whether the strain can grow without uracil supplementation (has the *ura4* pUR18 plasmid) or cannot grow (no plasmid). The upper two sectors do not grow indicating loss of the plasmid while the other four sectors do grow indicating the plasmid is present. When these same single colonies were tested on repressing plates supplemented with uracil (left, lower plate in FIG. 25), all six grew as expected, but all six also grew equally well on the inducing plate (right, lower plate in FIG. 25). This shows that the plasmid in this strain does not carry a suppressor. A second strain was found in this screen that had identical properties.

[0165] Since these two strains do not have a plasmid suppressor, the protease gene in these strains was amplified by PCR and sequenced. Both protease genes have a frameshift mutation that removes the amino acids after codon 18 of the protease (FIG. 26), a mutation that would be expected to eliminate protease activity. Both of the frameshift mutations occur in a section of 6 G's at codon 16 and 17 of the wild-type sequence, with one frameshift adding a G and one deleting a G. The occurrence of both frameshift mutations at the same place indicates that this run of 6 G's is a hotspot for mutations. Furthermore, the rate of spontaneous mutations in the protease gene is the minimal background that can be achieved in screening for suppressors, and the isolation of two spontaneous mutations indicates that the screening procedure is approaching this minimal background. By incorporating plasmid loss into the screening procedure, the background is low enough that during the screening of a genomic library only the one false positive described below was found. Concerning the background in screening for plasmid suppressors: in screening 3×10^5 cells, there were found only two G418^r strains with strong growth on -T; the strong growth did not require the plasmid and both had a frame shift mutation in the protease gene; and after completing the screening procedure through plasmid loss, the only false positive was one strain with weak growth on -T where the isolated plasmid did not show any suppression when retested in yeast.

EXAMPLE 5

EXEMPLARY AUTHENTIC WEAK SUPPRESSOR PLASMIDS CARRY THE HHP2 GENE

[0166] After completing the screening procedure through plasmid loss, eleven strains that seemed to be weak suppressors of the protease were isolated. The re-screening on inducing plates of colonies from the G418 replica is shown in FIG. 27 for six of these weak suppressors (labeled PS3, PS4 PS5, PS6 and PS7). These six weak suppressor strains grow well on G418 (top plate) and repressing plates (left, bottom plate) and grew slowly on inducing plate (right, bottom) requiring 6 days incubation, rather than 3 to 4 days, to give medium size colonies. This weak suppression of the protease is dependent on the plasmid as shown by plasmid loss for PS4 in FIG. 28. Four single colonies after growth of PS4 on nonselective media, labeled PS4-1, PS4-2, PS4-3 and PS4-4, were analyzed on media selective for the plasmid (top plate), and two, PS4-1 and PS4-2, had lost the plasmid and two, PS4-3 and PS-4, had retained the plasmid. All four grew on the repressing plate on the bottom, left, but only the two with the plasmid, PS4-3

and PS-4, had many colonies growing up on the inducing plate while most cells from the two without the plasmid, PS4-1 and PS4-2, did not grow on the inducing media.

[0167] To confirm the presence of a suppressor on the plasmid in these eleven strains, DNA was prepared from them and used to transform *E. coli* selecting for ampicillin resistance to recover the plasmid. As a final confirmation of a suppressor being carried on the plasmid, the recovered plasmid was transformed back into PrIntA and the transformants were retested for suppression. For PS3 and PS4 yeast suppressor strains from which the plasmids pPS3-2 and pPS4-10 were recovered in *E. coli*, two transformants of PrIndA were tested. The pPS3-2 plasmid does not re-test as a suppressor of the protease, as the growth of both transformants is identical to the transformants with the vector pUR18 (FIG. 29). PS3 is the only strain that passed through all steps of the screening process including plasmid loss, but whose recovered plasmid did not re-test as a suppressor when transformed back into PrIntA. For pPS4-10, both transformants show some growth in inducing media which is clearly more growth than seen for the vector pUR18 and pPS3-2 transformants (FIG. 29). The pPS4-10 plasmid then does carry a weak suppressor of the protease. The other nine plasmids recovered from weak suppressor yeast strains also show some growth in inducing media (FIG. 30). When tested for effect on plating efficiency 24 hr after induction, transformants with pPS3-2 and the vector pUR18 had low plating efficiencies of around 0.4 %, but transformants with the other ten recovered plasmids had plating efficiencies around 7% (FIG. 31).

[0168] The gene carried on these ten suppressor plasmids was identified by sequencing about 600 nucleotides for each end of the insert in plasmid, and it was found that they carried only the *hhp2* gene (FIG. 32). Each of the ten plasmids had the entire *hhp2* ORF and did not have any of the ORF for the genes adjacent to *hhp2*. Of these ten *hhp2* plasmids, six different plasmids were isolated from the 40,000 transformants screened from the genomic library, and these different plasmids came from independent transformants. The three cases where the same plasmid was isolated more than once were from the same transformation pool and are probably repeated isolations of the same transformant. The weak suppressor plasmids all contain the *hhp2* gene: ten weak suppressor strains passed all steps of the screening through plasmid loss; plasmids isolated from these ten strains all retested as weak suppressors when transformed back into yeast; all ten plasmids contain the *hhp2* gene and no other gene; and the ten plasmids include six different *hhp2* plasmids.

[0169] Because *hhp2* was isolated from six independent transformation pools and no other gene was identified, the *hhp2* gene may be the only gene in the pUR18 genomic library that suppresses the protease sufficiently to be detected in this screen. It is worth noting that the *hhp1* gene was not found in the screen for protease suppressors. The *hhp1* and *hhp2* genes are closely related (74% identity) and have partially overlapping functions (Dhillon and Hoekstra 1994).

[0170] The identification of *hhp2* as the suppressor gene rules out one possible explanation for its suppressor activity. High level expression of the protease may be toxic to the yeast cells because an essential protein may be a substrate for the protease, and digestion of this essential protein by the protease lowers its level to the point where there is not enough for the cell to remain viable. In this model, higher level expression of the gene for this essential protein would be expected to reduce the toxic effects of protease expression. However, this model does not apply to *hhp2* since a strain with a deletion of *hhp2* is viable and grows well (Dhillon and Hoekstra 1994). The failure to isolate any other gene that suppresses the effects of protease expression indicates that in one embodiment there is more than one essential protein that is a substrate for the protease or in an alternative embodiment there is some other mechanism for protease toxicity.

[0171] The actual mechanism by which *hhp2* suppresses the protease may be further characterized. The Hhp2 kinase belongs to the CK1 class of kinases (Dhillon and Hoekstra 1994), and there have been no reports of interactions between this class of kinases and HIV-1 protease. In certain aspects of the invention, the role that the kinase activity of Hhp2 plays in the suppression of the protease is characterized. One embodiment is that Hhp2 inhibits the protease by phosphorylation. Another embodiment is that the kinase does not phosphorylate the protease but that Hhp2 is a substrate which acts as a competitive inhibitor of the protease. In aspects of the invention, it is established whether *hhp2* defines a new mechanism to inhibit HIV-1 protease.

[0172] The identification of *hhp2* is also a direct demonstration that the screening procedure developed here is sufficiently sensitive to find rare suppressors. The *hhp2* gene is one of about 5000 genes in fission yeast (Wood, Gwilliam *et al.* 2002), and the screen was able to find this one gene on six different plasmids. This result indicates that this exemplary screening procedure has been developed to the point where it can be used to isolate rare protease suppressors from other libraries expressing even more proteins and peptides.

REFERENCES

[0173] All patents, patent applications, and publications mentioned in the specifications are indicative of the levels of those skilled in the art to which the invention pertains. All patents, patent applications, and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

PATENTS AND PATENT APPLICATIONS

- [0174] U.S. Patent No. 5,384,253
- [0175] U.S. Patent No. 5,399,363
- [0176] U.S. Patent No. 5,466,468
- [0177] U.S. Patent No. 5,543,158
- [0178] U.S. Patent No. 5,580,579
- [0179] U.S. Patent No. 5,629,001
- [0180] U.S. Patent Nos. 5,641,515
- [0181] U.S. Patent No. 5,725,871
- [0182] U.S. Patent Nos. 5,756,353
- [0183] U.S. Patent No. 5,780,045
- [0184] U.S. Patent No. 5,804,212
- [0185] U.S. Patent No. 5,792, 451
- [0186] U.S. Patent No. 6,043,357
- [0187] U.S. Patent No. 6,613,308
- [0188] Statutory Invention Registration H1,649

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[0200] Wood, V., R. Gwilliam, *et al.* (2002). "The genome sequence of *Schizosaccharomyces pombe*." *Nature* 415(6874): 871-80.

[0201] Zurlinden, A. and M. E. Schweingruber (1997). "Identification of a DNA element in the fission yeast *Schizosaccharomyces pombe* *nmt1* (*thi3*) promoter involved in thiamine-regulated gene expression." *J Bacteriol* 179(18): 5956-8.

[0202] Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined by the appended claims. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, composition of matter, means, methods and steps described in the specification. As one of ordinary skill in the art will readily appreciate from the disclosure of the present invention, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the present invention. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.

CLAIMS

What is claimed is:

1. A method of identifying an HIV protease inhibitor, comprising:

providing a yeast cell, wherein said yeast cell comprises a polynucleotide comprising sequence encoding HIV protease;

subjecting the polynucleotide to a candidate agent; and

assessing one or both of the following characteristics:
 - 1) the viability and/or growth of said yeast cell, wherein the ability to survive in the presence of said candidate agent, as compared to in the absence of said candidate agent, identifies said candidate agent as said HIV protease inhibitor; and
 - 2) cleavage of an HIV protease substrate, wherein the ability of the candidate agent to inhibit the cleavage of the substrate identifies said candidate agent as said HIV protease inhibitor.
2. The method of claim 1, wherein said HIV protease is HIV-1 protease.
3. The method of claim 1, wherein said HIV protease is HIV-2 protease.
4. The method of claim 1, wherein expression of said HIV protease is under the regulation of an overexpression regulatory sequence.
5. The method of claim 1, wherein said inhibitor is a polypeptide, a polynucleotide, a small molecule, an antibody, or a mixture or combination thereof.
6. The method of claim 1, wherein said yeast is a fission yeast.

7. The method of claim 6, wherein said fission yeast is *Schizosaccharomyces pombe*.
8. The method of claim 1, wherein said polynucleotide is incorporated into the genome of the yeast.
9. The method of claim 4, wherein said overexpression regulatory sequence comprises a *nmt1* overexpression regulatory sequence, an *adh1* regulatory sequence, a *fbp1* regulatory sequence, an *inv1* regulatory sequence, or a *ctr4* regulatory sequence.
10. The method of claim 1, wherein the polynucleotide is further defined as comprising at least one moiety for reducing background growth in a viability screen.
11. The method of claim 10, wherein said moiety for reducing background comprises a marker.
12. The method of claim 11, wherein said marker comprises resistance to an antibiotic.
13. The method of claim 11, wherein said marker comprises resistance to G418 or hygromycin.
14. The method of claim 11, wherein said marker is a nutritional marker.
15. The method of claim 11, wherein said marker comprises *ade1*, *ade6*, *arg3*, *CAN1*, *his3*, *his7*, *leu1*, *leu2*, *sup3-5*, *ura4*, and *ura3*.
16. The method of claim 1, wherein said HIV protease is a human HIV protease.
17. The method of claim 1, wherein said HIV protease substrate is a human HIV protease substrate.

18. The method of claim 1, wherein said assessment of cleavage of an HIV protease substrate comprises assessing cleavage of a polypeptide comprising a first polypeptide moiety and a second polypeptide moiety, wherein an HIV protease cleavage site is present between said first and second polypeptide moieties.
19. The method of claim 18, wherein the first polypeptide moiety comprises a detectable label.
20. The method of claim 18, wherein the second polypeptide moiety comprises a substrate for HIV protease.
21. The method of claim 20, wherein the substrate for HIV protease is HIV-1 Vpr polypeptide.
22. The method of claim 1, wherein the cleavage site comprises DSQNYPIVQ (SEQ ID NO:3), DSFNFPQIT (SEQ ID NO:4), VSQNYPIVQN (SEQ ID NO:8), KARVLAEAMS (SEQ ID NO:9), SATIMMQRGN (SEQ ID NO:10), RPGNFLQSRP (SEQ ID NO:11), VSPNFPQITL (SEQ ID NO:12), CTLNFPISPI (SEQ ID NO:13), GAETFYVDG (SEQ ID NO:14), or IRKVLFLDGI (SEQ ID NO:15).
23. The method of claim 19, wherein said detectable label comprises green fluorescence protein, emerald green fluorescence protein, yellow fluorescence protein, blue fluorescence protein or cyan fluorescence protein.
24. The method of claim 1, further comprising delivering a therapeutically effective amount of said inhibitor to an individual that has been infected with the HIV virus or that has AIDS.
25. The method of claim 1, further comprising delivering an effective amount of said inhibitor to an individual at risk for becoming infected with HIV or at risk for developing AIDS.

26. A method of treating an individual infected with HIV virus or that has AIDS, comprising the step of delivering to the individual a therapeutically effective amount of a composition comprising *hhp2*.
27. The method of claim 26, wherein said *hhp2* is a polypeptide.
28. The method of claim 26, wherein said *hhp2* is a polynucleotide encoding a *hhp2* polypeptide.
29. A yeast cell, comprising a polynucleotide encoding HIV protease.
30. The yeast cell of claim 29, wherein the HIV protease is HIV-1 protease.
31. The yeast cell of claim 29, wherein the HIV protease is HIV-2 protease.
32. The yeast cell of claim 29, wherein expression of said polynucleotide is under the control of an overexpression regulatory sequence.
33. The yeast cell of claim 29, wherein said polynucleotide further comprises a marker.
34. The yeast cell of claim 33, wherein said marker is further defined as a marker for resistance to an antibiotic.
35. The yeast cell of claim 33, wherein said marker is further defined as a nutritional marker.
36. The yeast cell of claim 29, further comprising a polynucleotide comprising a first sequence encoding a first polypeptide moiety and a second sequence encoding a second polypeptide moiety, wherein a cleavage site for the protease is present between said first and second polypeptide moieties.

37. The yeast cell of claim 36, wherein the first polypeptide moiety comprises a detectable label.
38. The yeast cell of claim 37, wherein the detectable label is green fluorescence protein, emerald green fluorescence protein, yellow fluorescence protein, blue fluorescence protein or cyan fluorescence protein.
39. The yeast cell of claim 36, wherein the second polypeptide moiety comprises HIV-1 Vpr polypeptide.
40. The yeast cell of claim 29, further defined as being in a culture of yeast cells or a colony of yeast cells.
41. A kit for the treatment and/or prevention of HIV infection and/or AIDS, comprising a polynucleotide encoding *hhp2* or a hhp2 polypeptide housed in a suitable container.
42. The kit of claim 40, further comprising a pharmaceutically acceptable excipient.
43. A kit for screening for one or more HIV protease inhibitors, comprising the yeast cell of claim 29.
44. The kit of claim 43, wherein said yeast cell further comprises a polynucleotide comprising a first sequence encoding a first polypeptide moiety and a second sequence encoding a second polypeptide moiety, wherein a cleavage site for the protease is present between said first and second polypeptide moieties.

Loss of integrated gene by homologous
recombination creates a high background in
suppressor screens

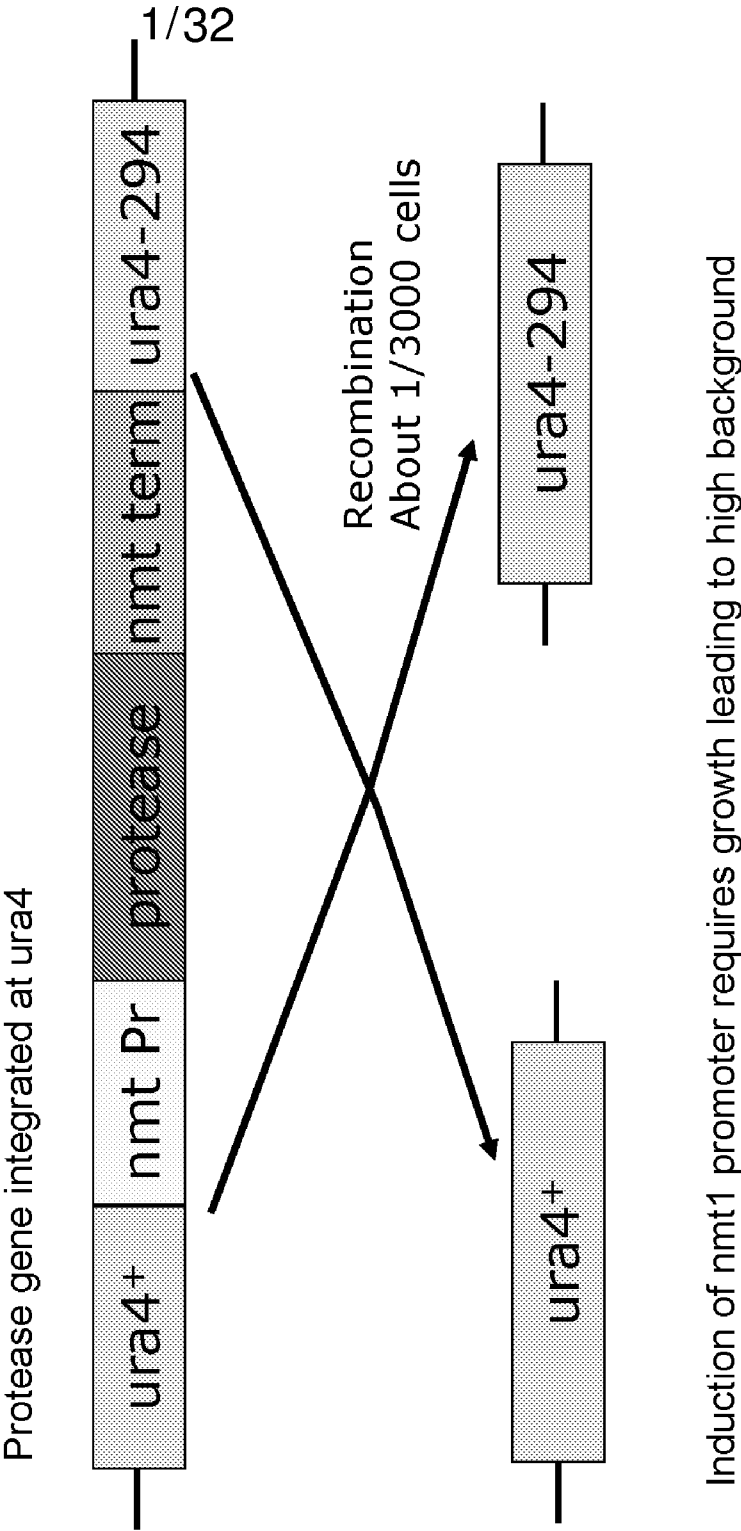


FIG. 1

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A kanr gene adjacent to the
protease gene for identifying loss
by homologous recombination

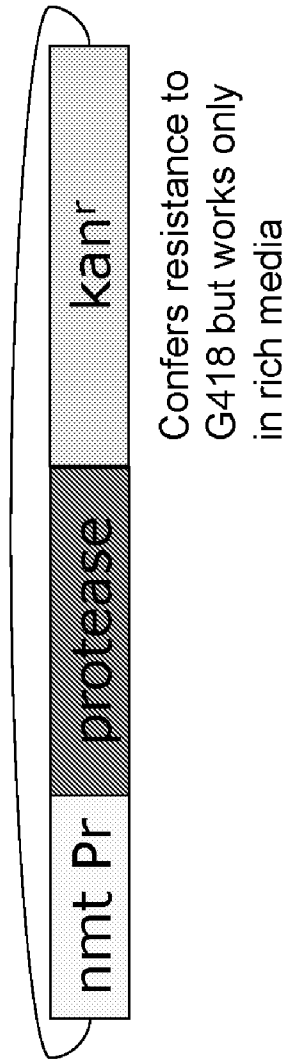
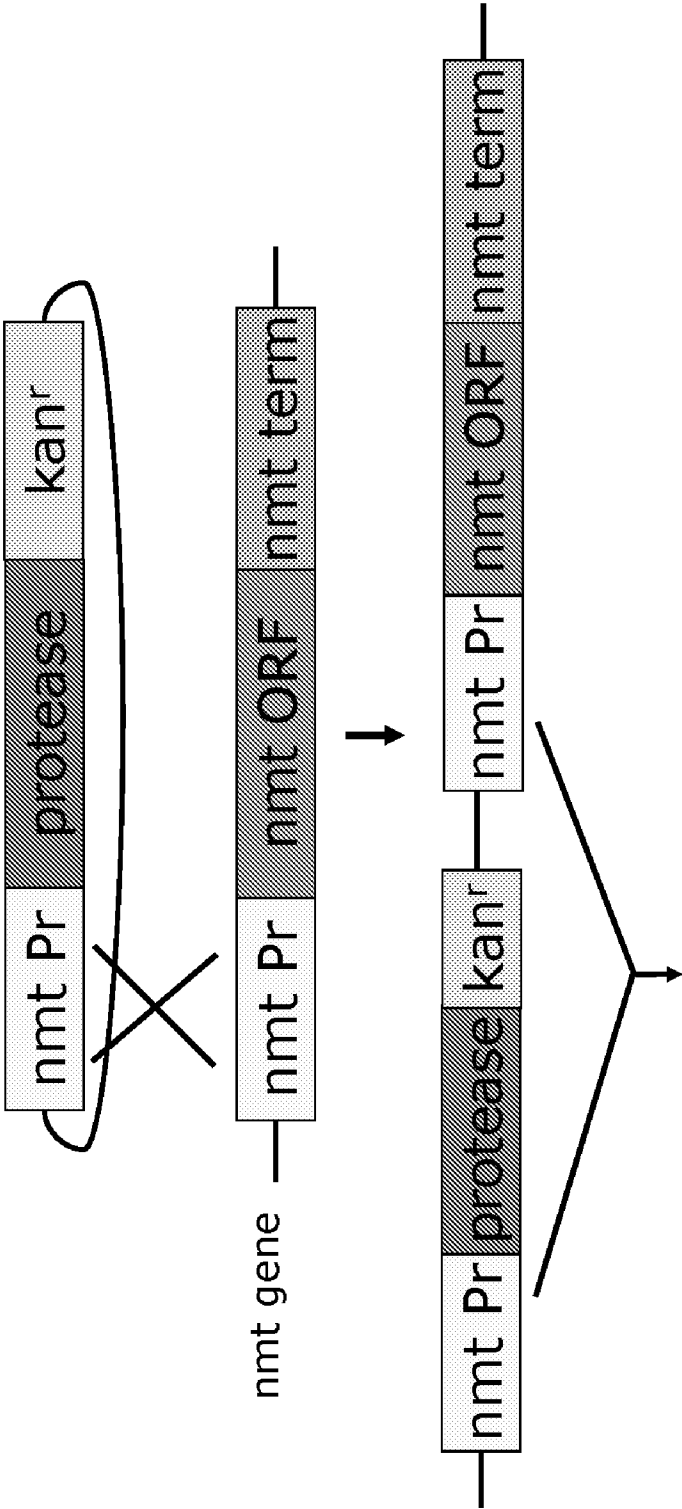


FIG. 2

Plasmid integrated at the nmt1 locus by homologous recombination



Recombination across nmt Pr causes loss of protease and kan^r

FIG. 3

Two G418^r strains were isolated and their growth is strongly inhibited when the protease gene is expressed

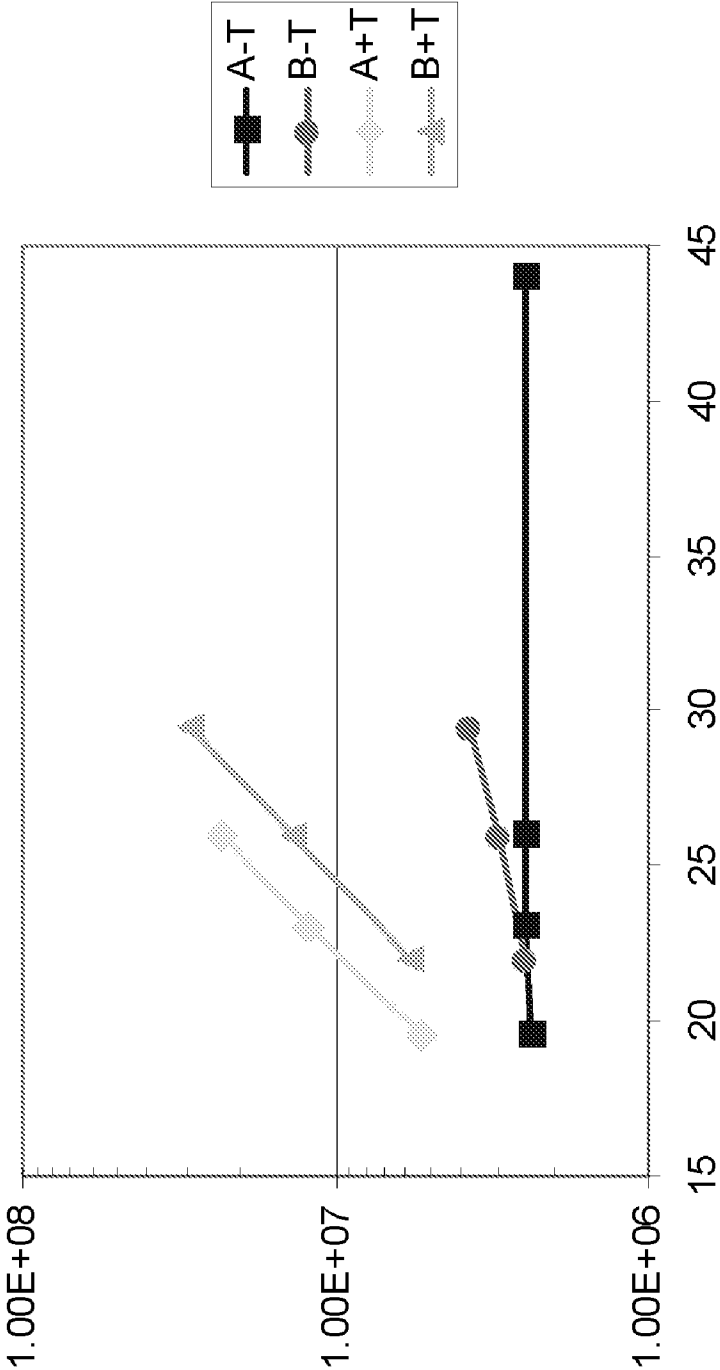


FIG. 4

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Cells on an inducing plate do not
form colonies

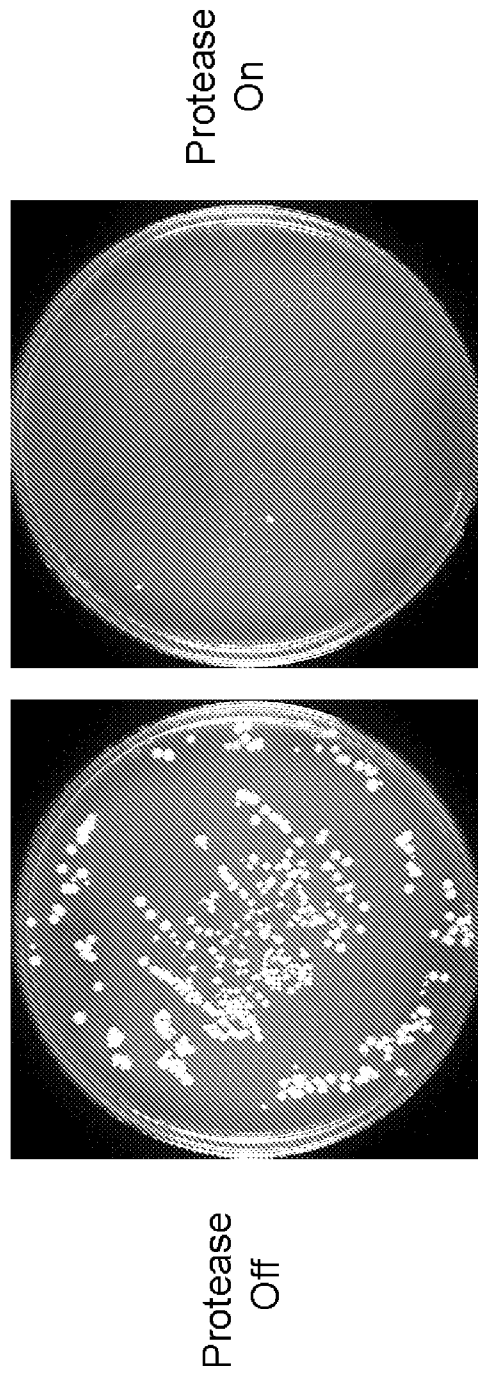


FIG. 5

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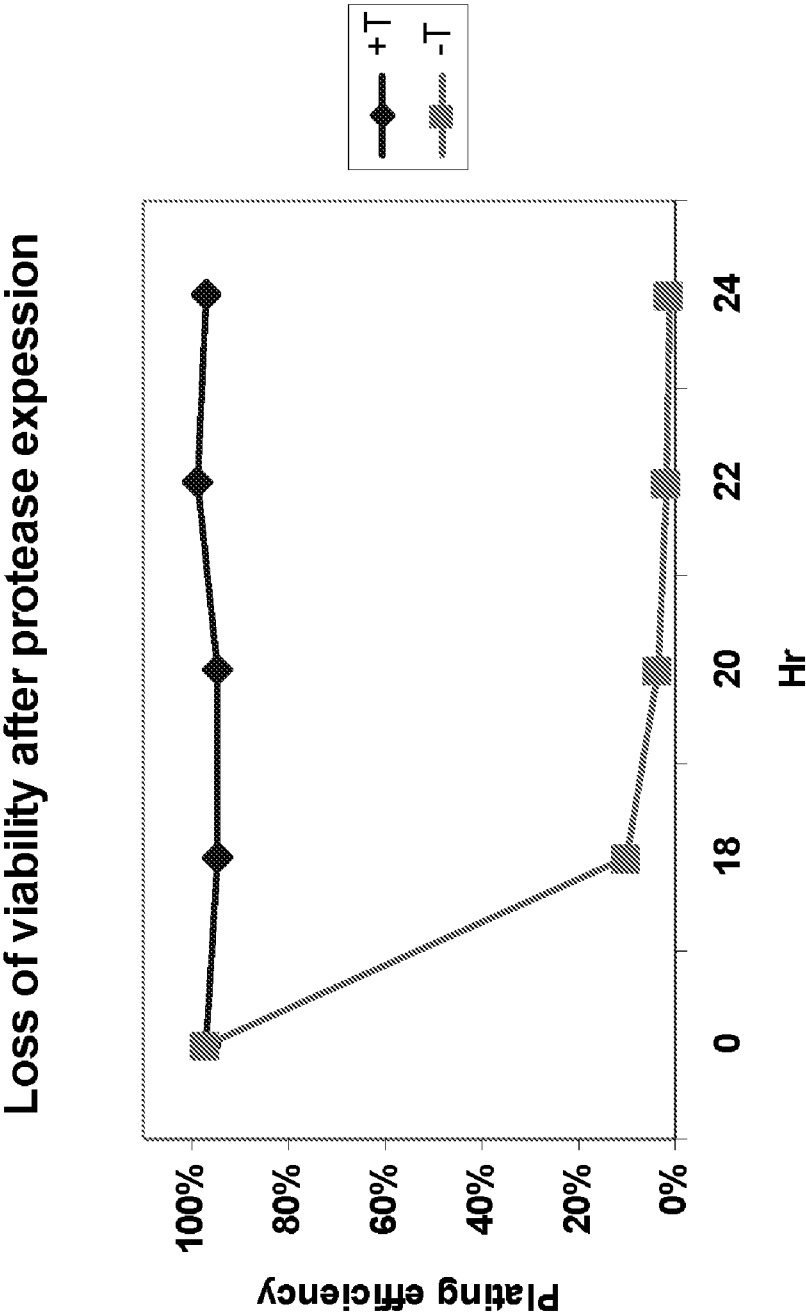


FIG. 6

Protease activity can be monitored
by relocation of a GFP-Vpr
fusion

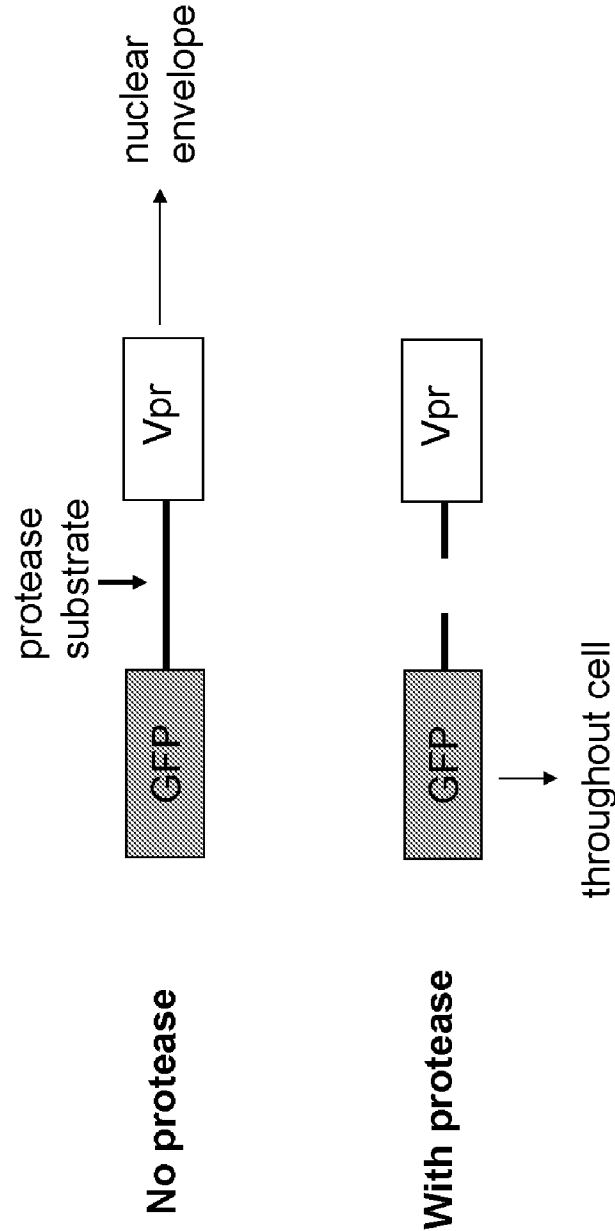
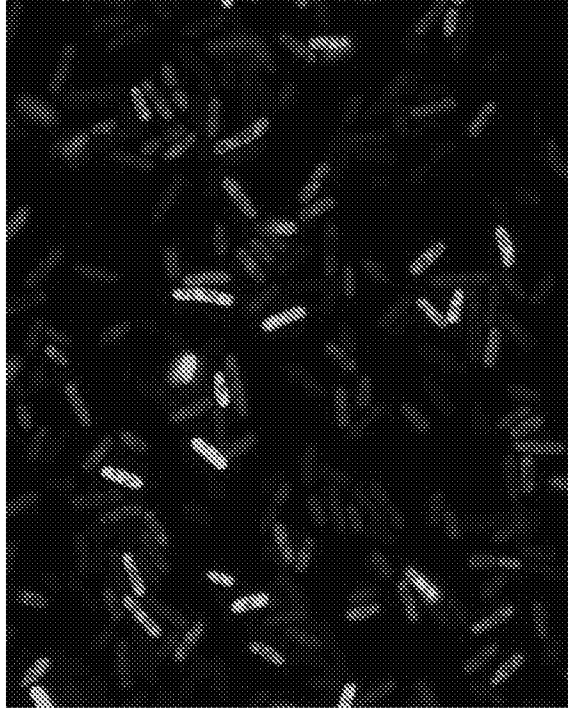


FIG. 7

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HIV-1 Protease does not affect localization of GFP

Protease



No Protease

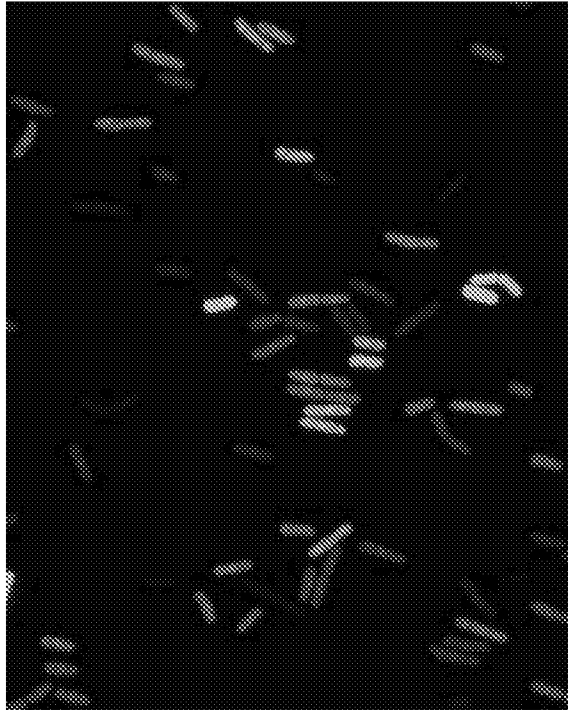
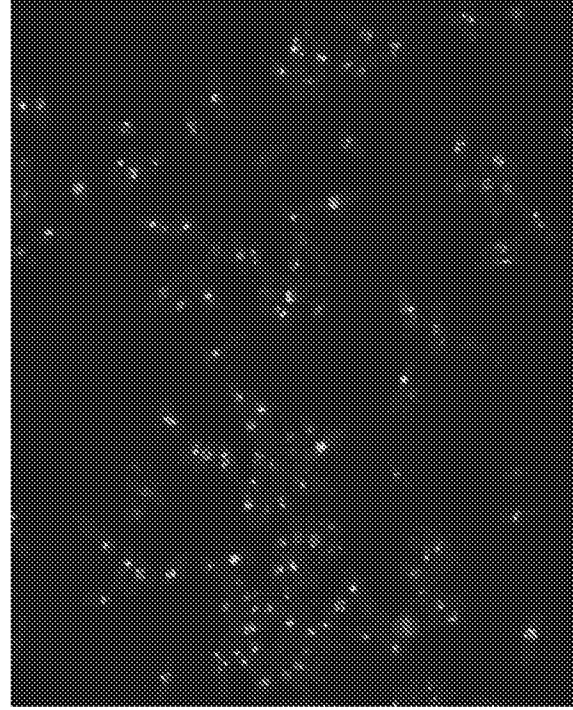


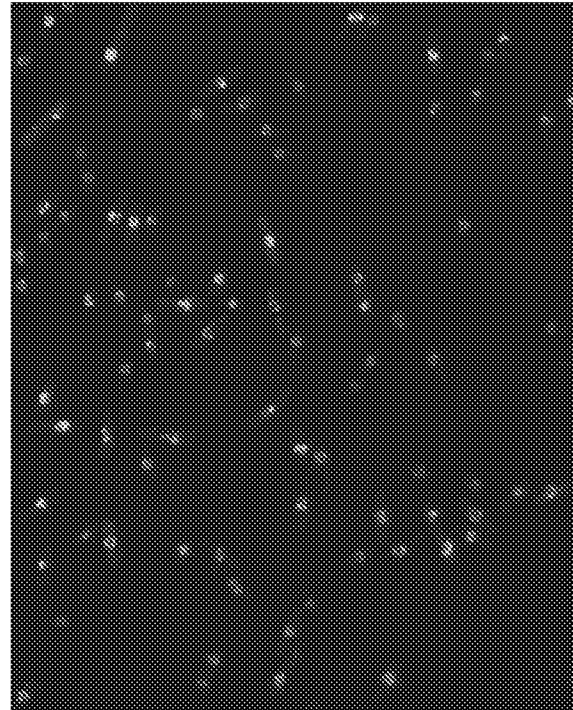
FIG. 8

Protease does not relocate GFP-Vpr to cytoplasm

Protease



No Protease



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FIG. 9

Protease relocates GFP in GFP-SL Ma-vpr to cytoplasm

No Protease

Protease

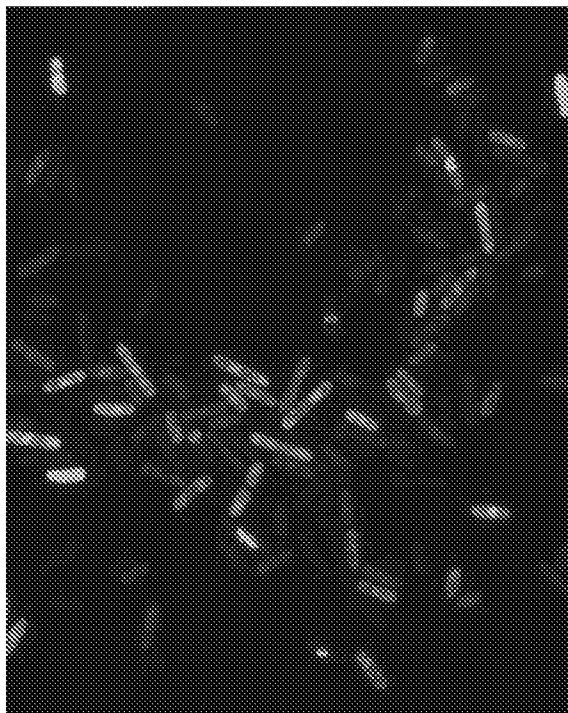
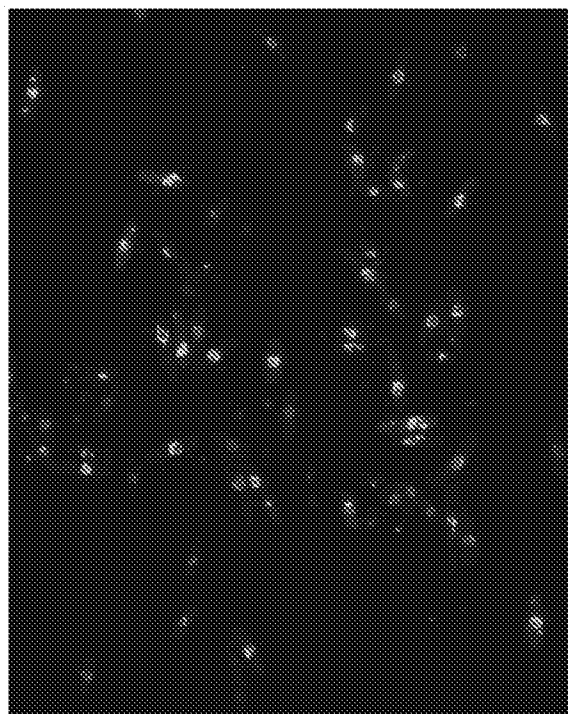


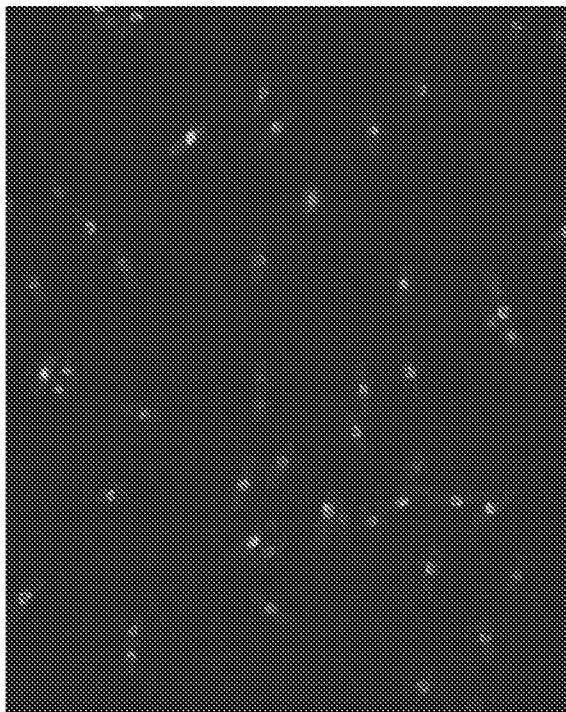
FIG. 10

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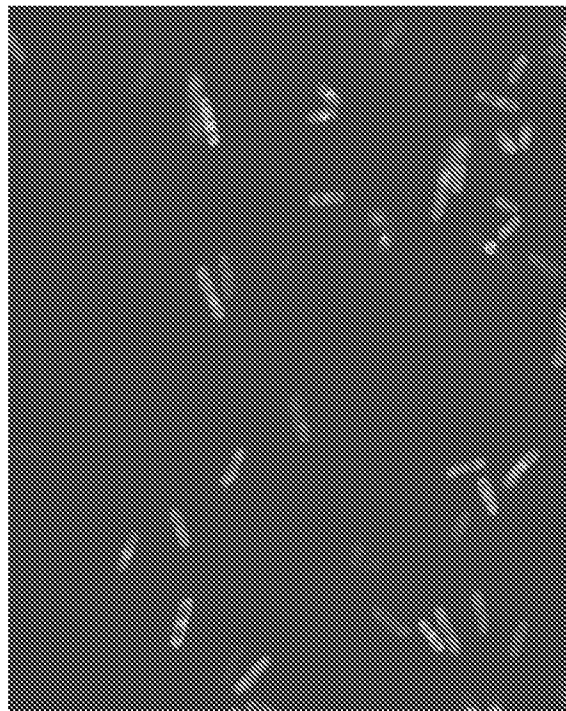
Substrate Linker MA (SL Ma) $\rightarrow$ DSQNYPIVQ Contains cleavage site for protease

Protease relocates GFP- SL p6-Vpr to cytoplasm

No Protease



Protease

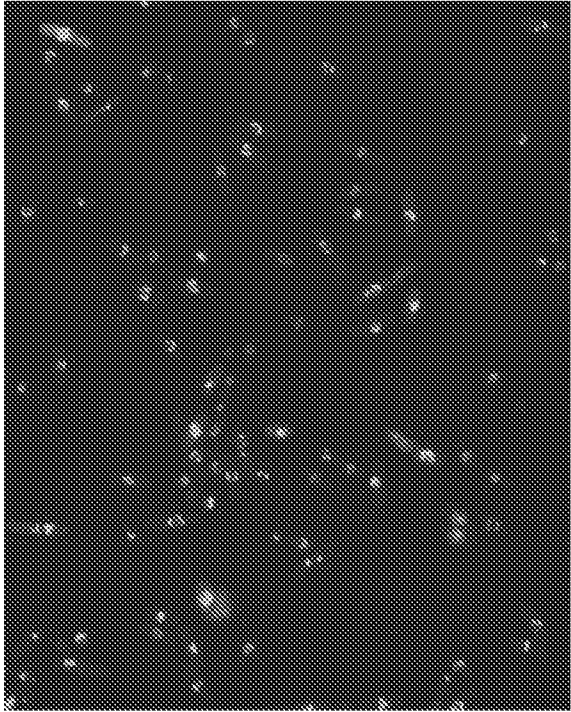


↓
Substrate Linker p6 (SL p6) DSFNFPQIT Contains cleavage site for protease

FIG. 11

Protease does not relocate GFP in GFP-SL A-Vpr to cytoplasm

No Protease



Protease

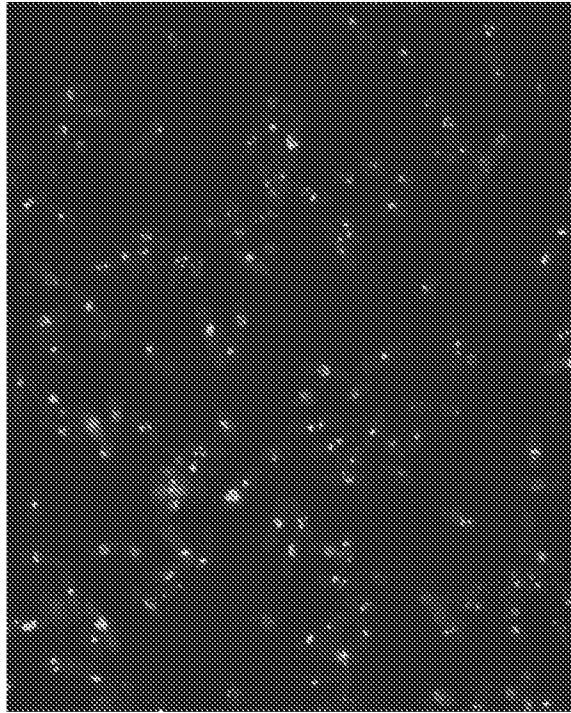
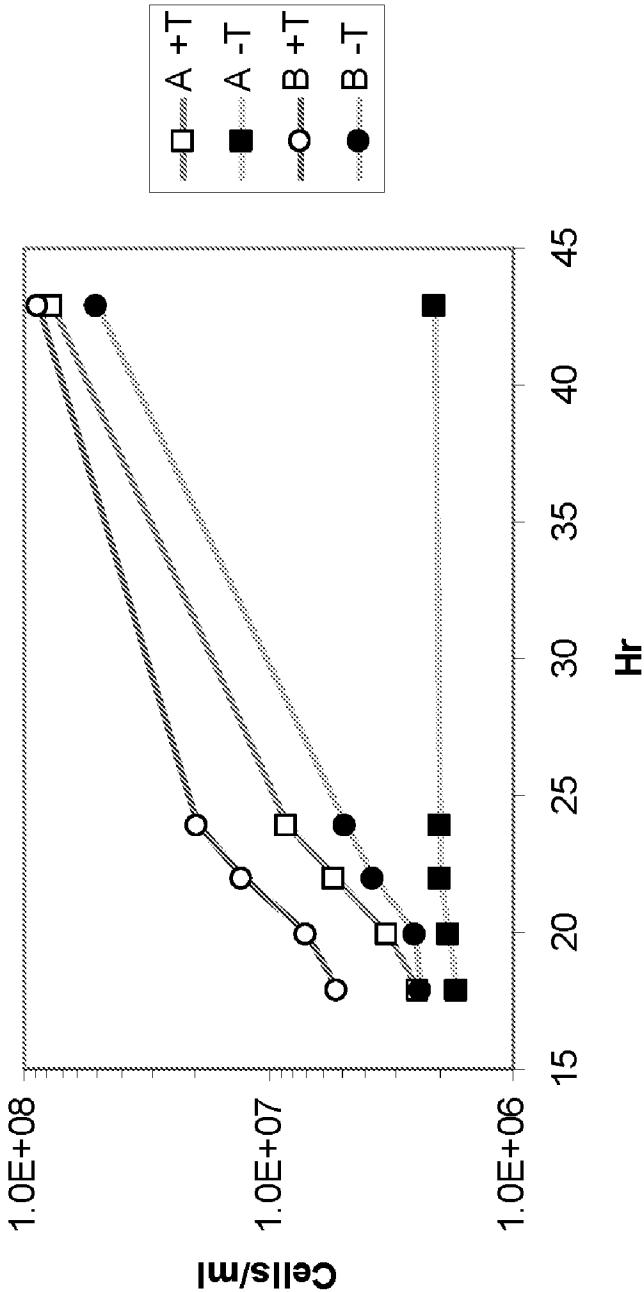


FIG. 12

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Substrate Linker A (SL A) KKKKVLPIELNAATDK No cleavage site for protease

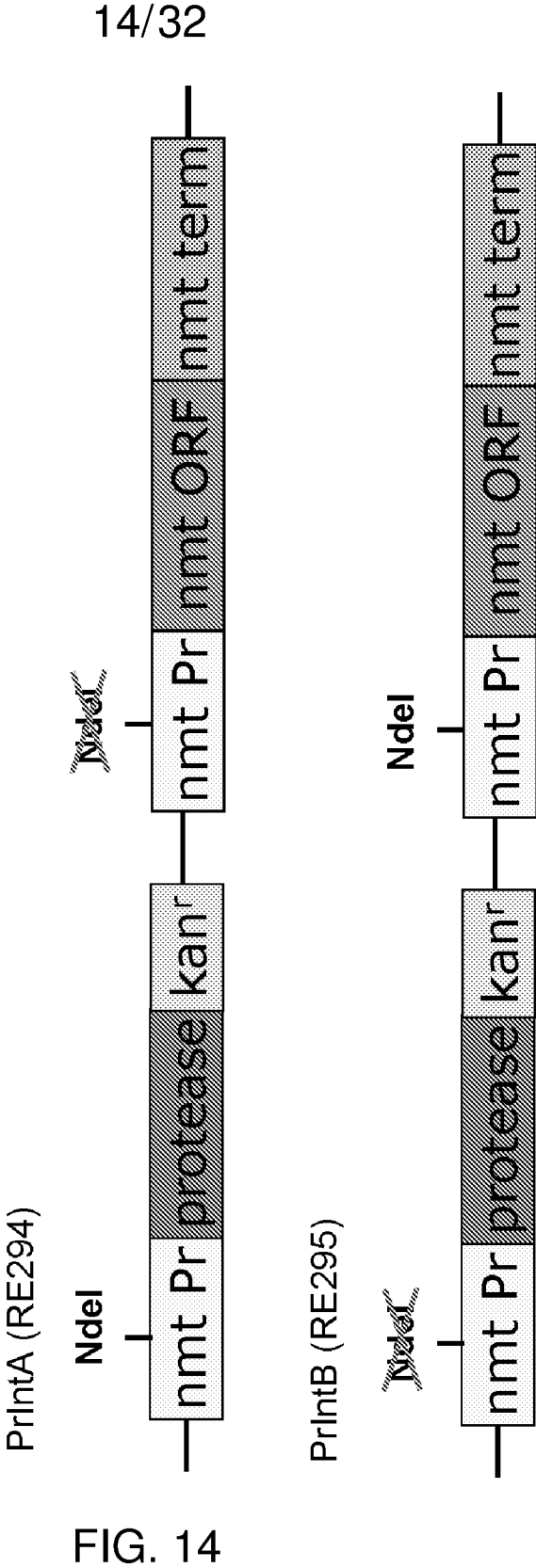
The PrIntA G418^r strain inhibits growth more than the PrIntB strain



PrIntA is RE294 and PrIntB is RE295

FIG. 13

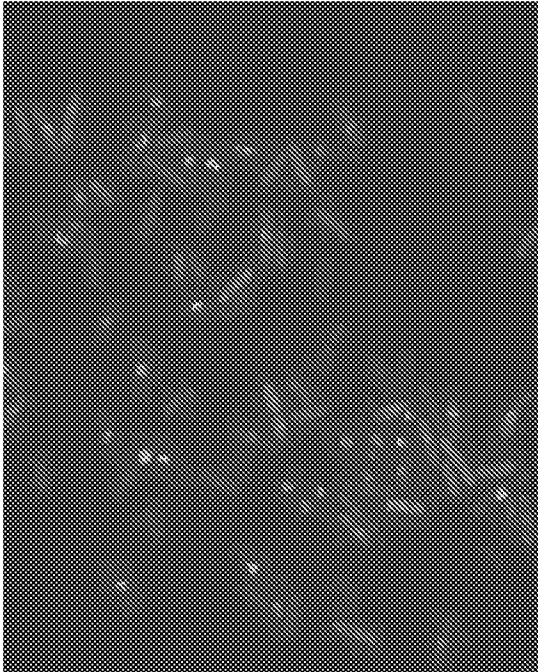
Both strains have a single plasmid integrated at the nmt1 locus but the sequence of the nmt1 promtor is different



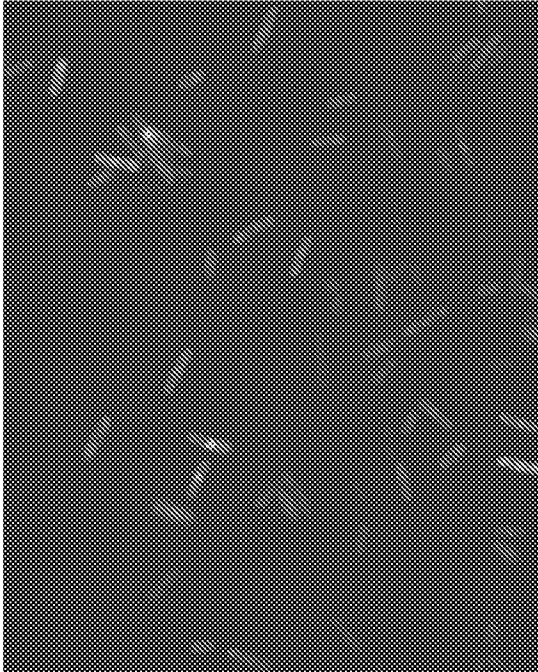
Difference results from exact point of cross over during homologous recombination

%GFP pattern indicates amount of
protease activity

PrIntB



PrIntA



Strain	% GFP pattern
PrIntA	85%
PrIntB	47%

FIG. 15

Indinavir suppresses effect of protease on growth

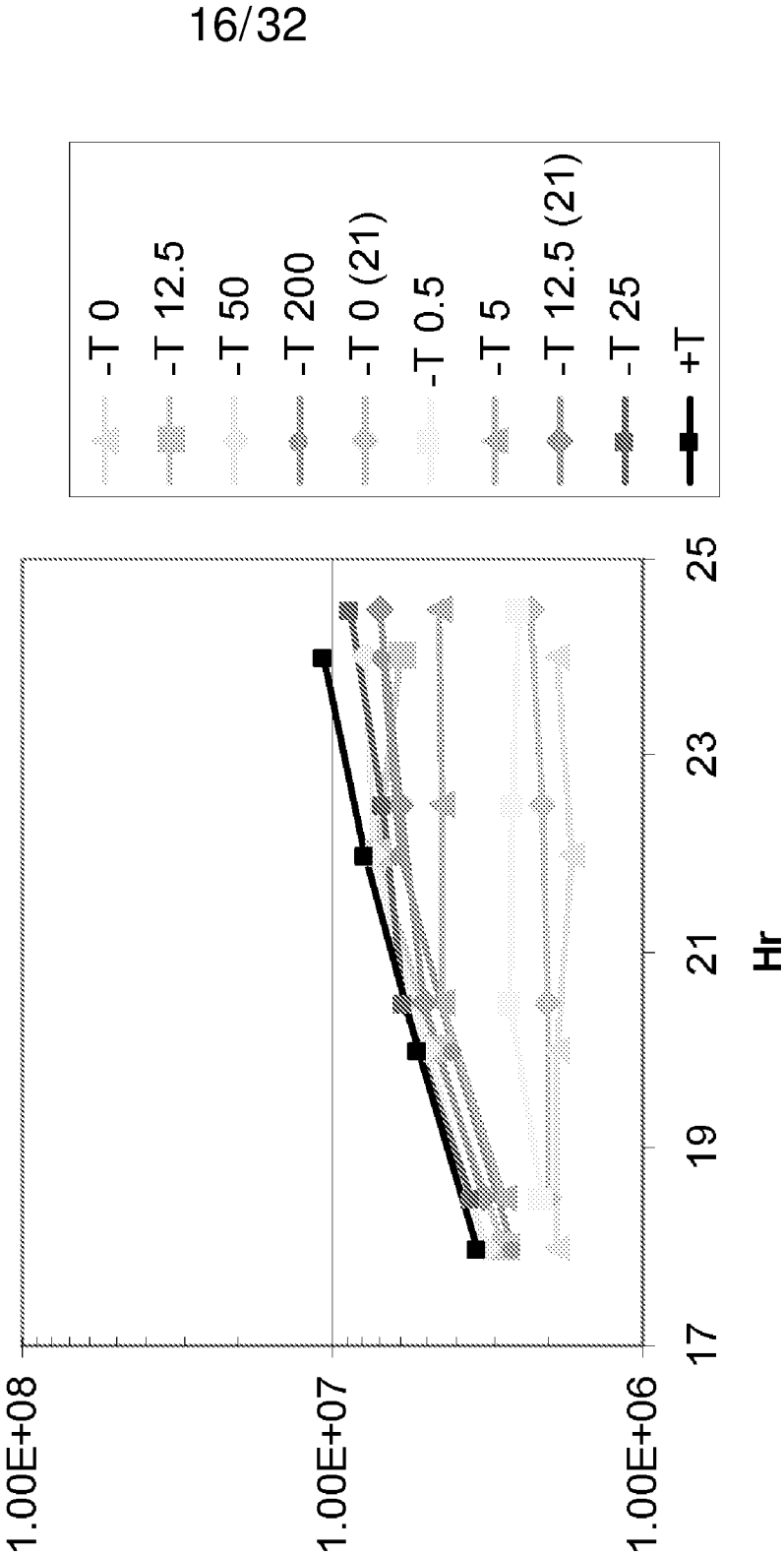


FIG. 16

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Indinavir prevents relocation of GFP in GFP-SL p6-vpr to cytoplasm

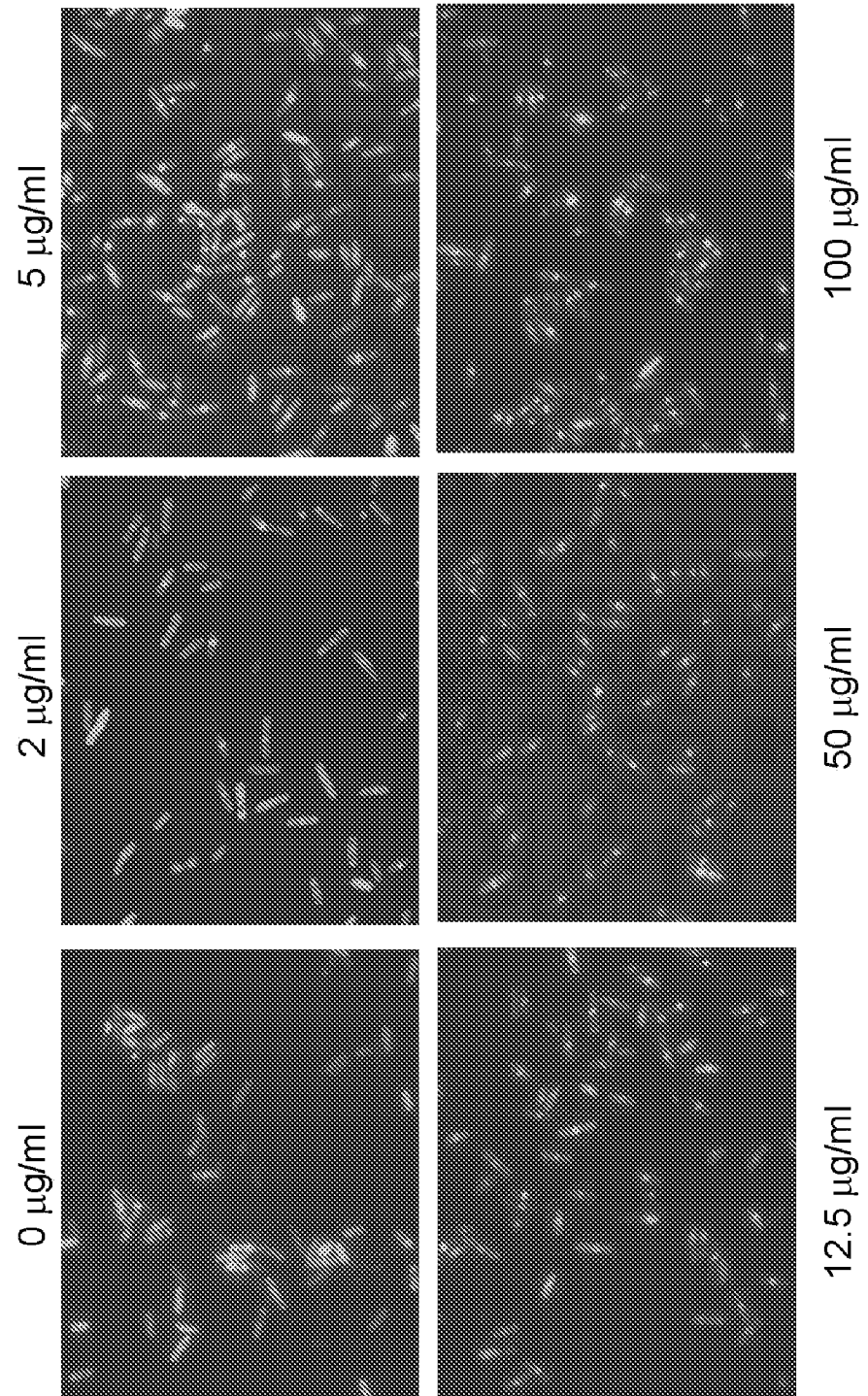


FIG. 17

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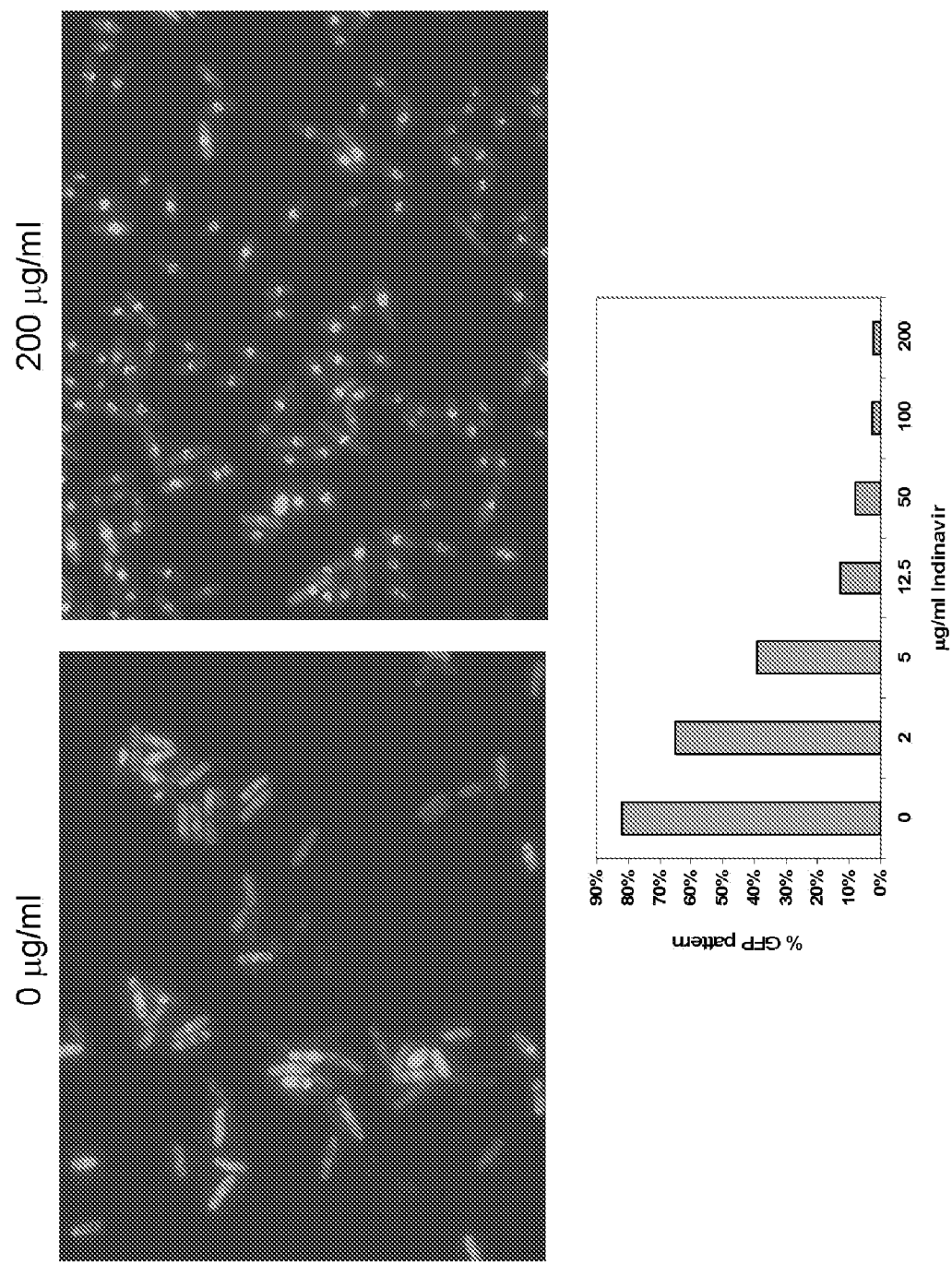


FIG. 18

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Indinavir allows colony formation

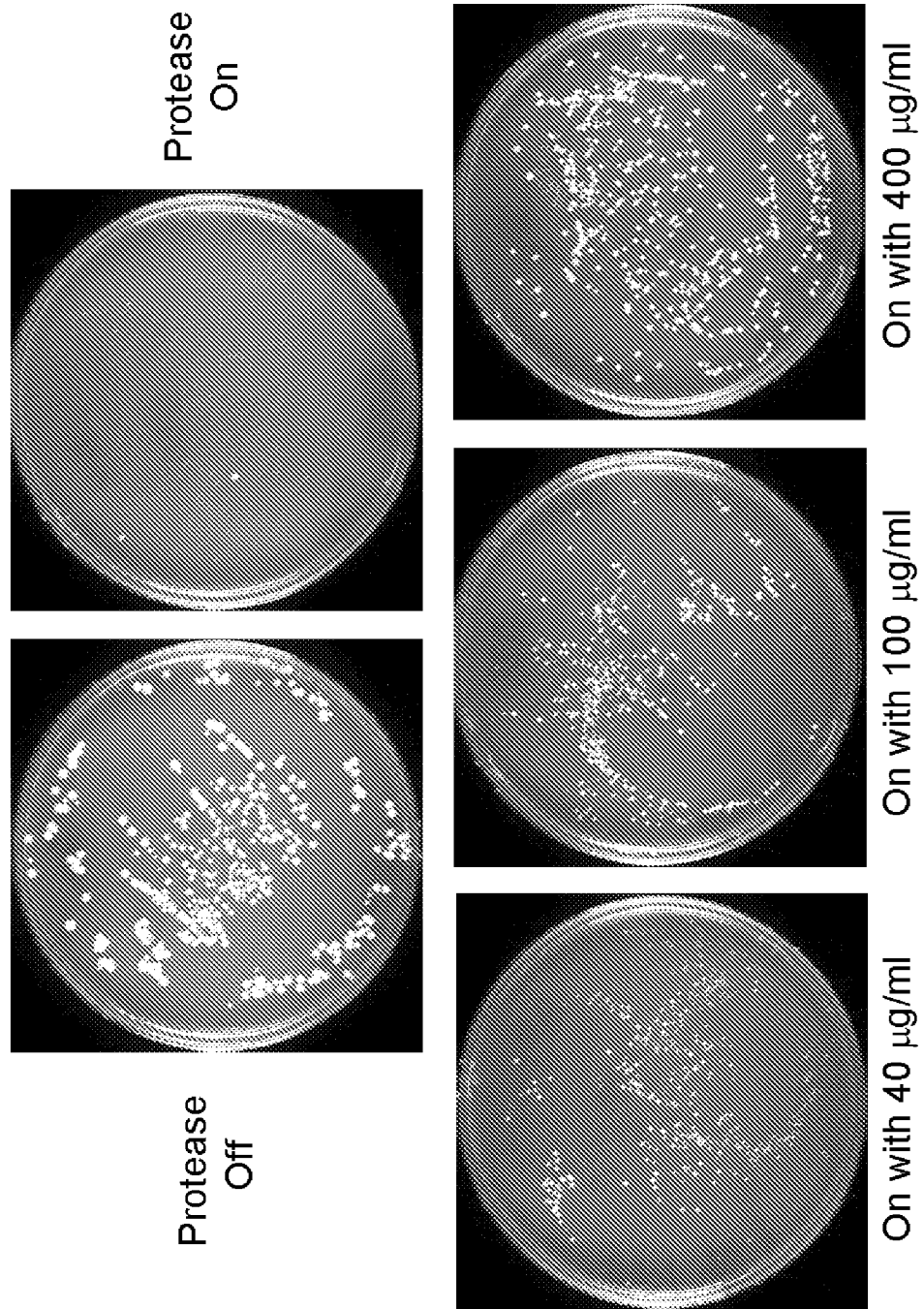
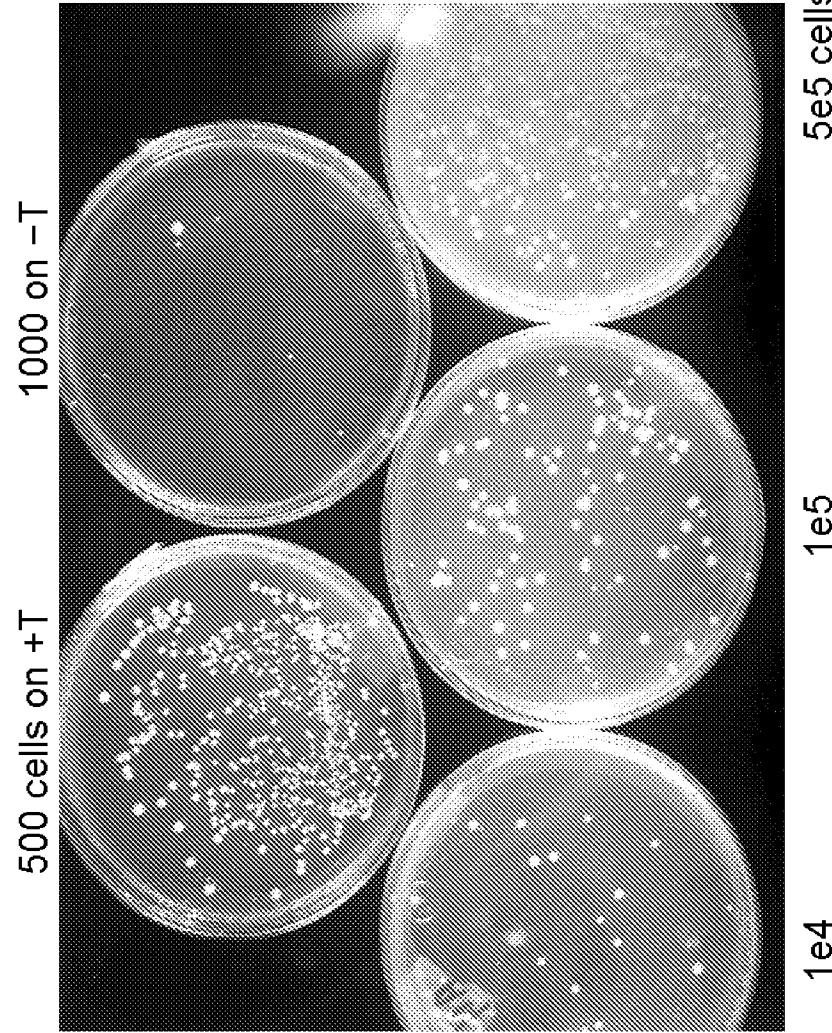


FIG. 19

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1. Only about 1 in 1000 cells forms a colony
on inducing plates

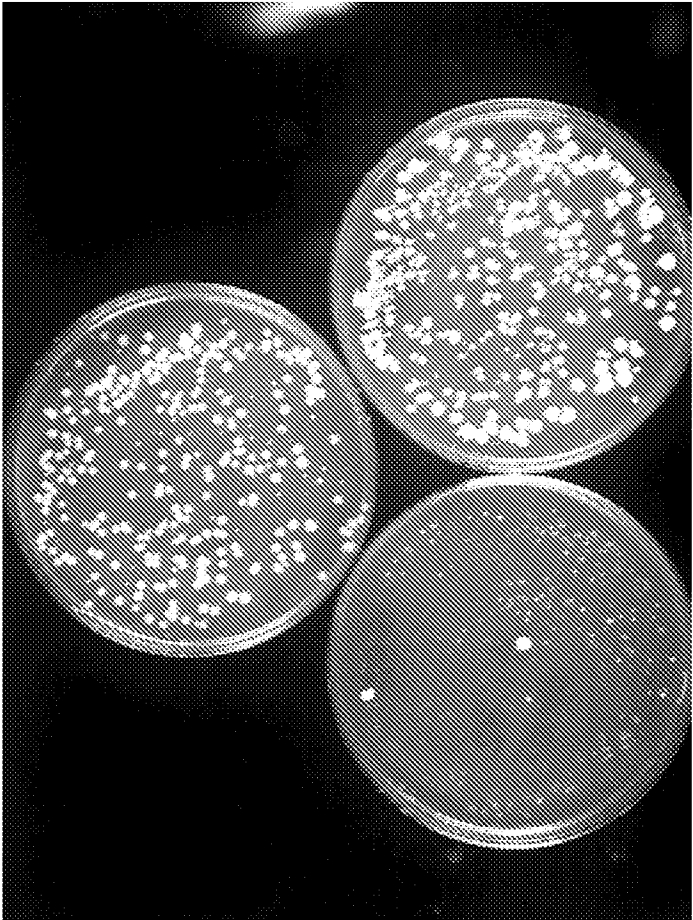


Frequency of colonies on inducing plates is consistent with plasmid integrated at nmt1 gene.

FIG. 20

2. Most colonies on the inducing plate have lost the kan^r gene

5e5 cells on -T



Replicas
incubated
for two days

FIG. 21

The protease and kan^r genes are lost through homologous recombination.

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3. When colonies from the G418 replica are retested on inducing plate, most still have the protease gene

G418^s prot⁻ G418^r prot⁺



Other six streaks are from colonies on G418 replicas.

Apparently the large colonies on the G418 replica result from a mixed colony on the inducing plate which contains both G418^r prot⁺ and G418^s prot⁻ cells. Enough G418^r prot⁺ cells are present in these rare colonies for growth on the G418 replica plate where expression of the protease gene is repressed.

FIG. 22

Low background in screening for protease suppressors with the PrIndA strain

Starting with the plating of 5e5 cells on an inducing plate, usually no colony is found which is actually resistant to the expression of the protease gene.

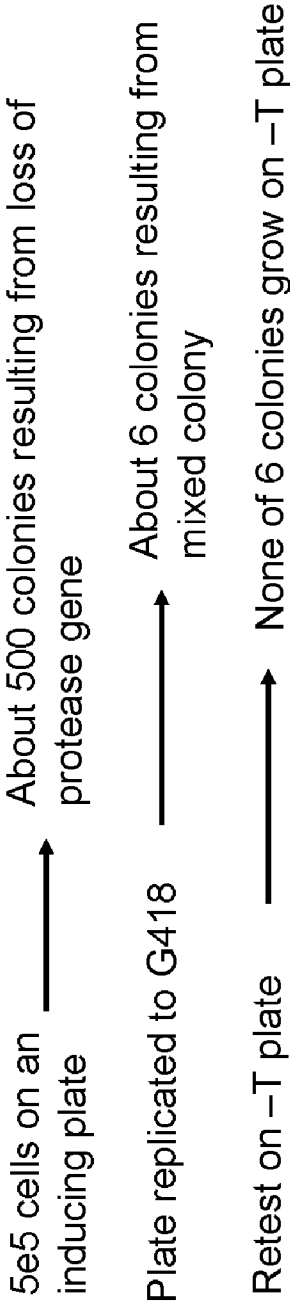


FIG. 23

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Example of background: G418^r and strong growth on -T

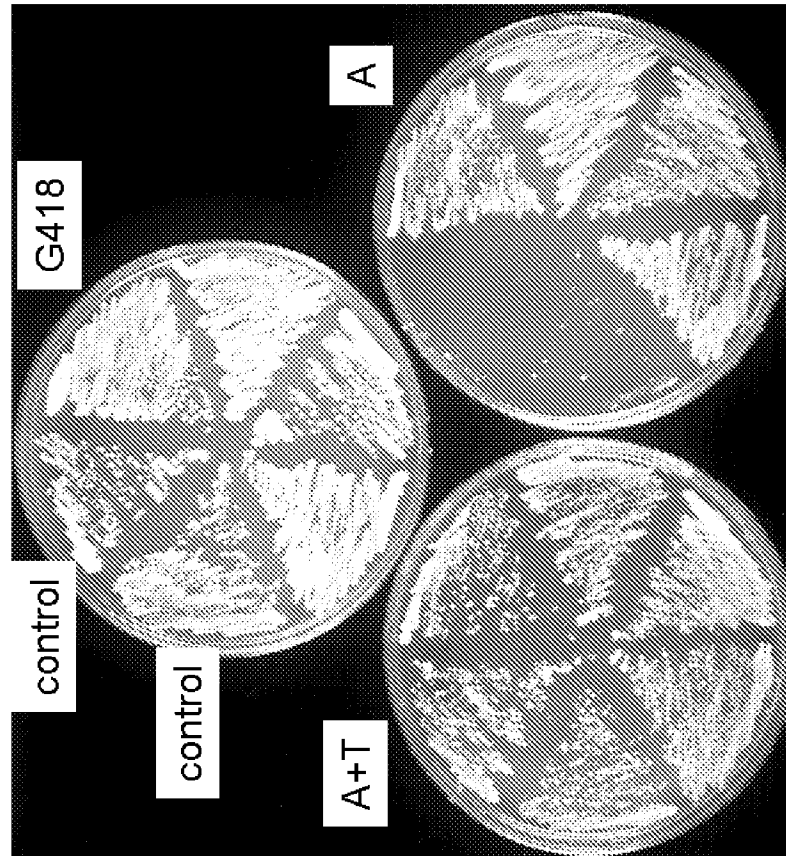
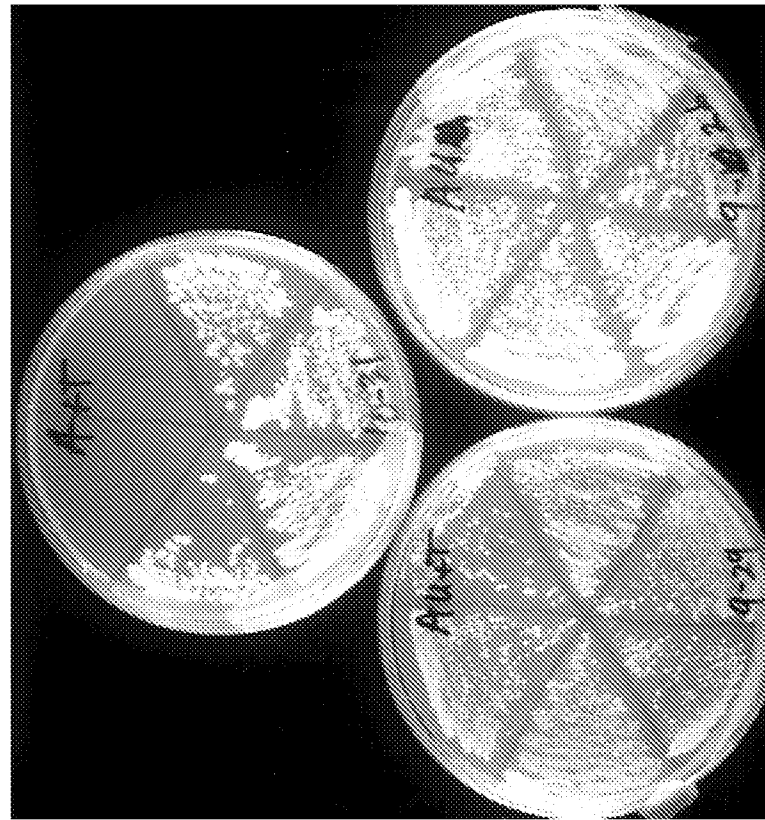


FIG. 24

Two control strains and four single colonies from a false positive suppressor strain.

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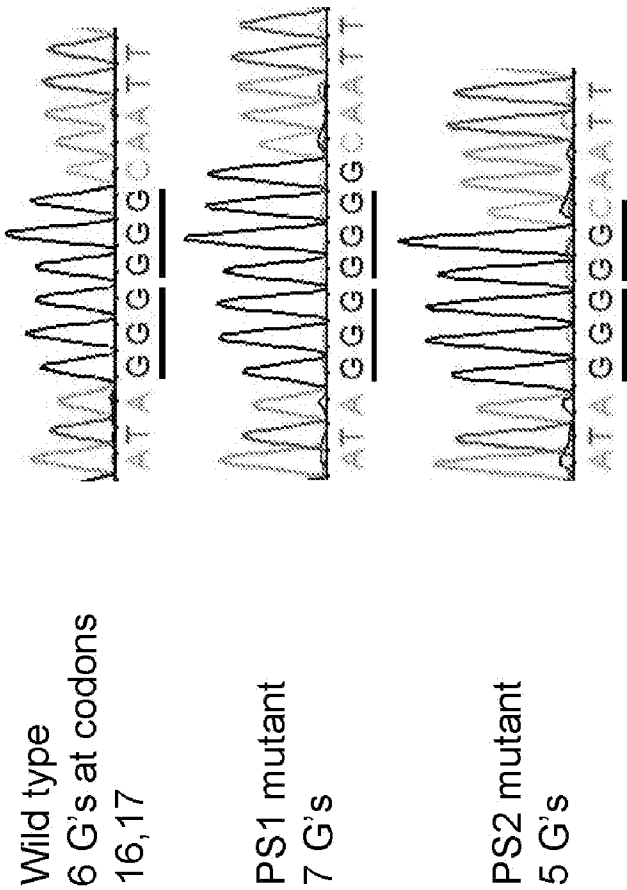
Strong growth on -T not dependent on plasmid



Of six colonies from the suppressor candidate grown on nonselective media, two had lost the pUR18 based plasmid, but still had strong growth on the -T plate.

FIG. 25

Both background strains with normal growth on inducing plates have a frame shift mutation in the protease gene

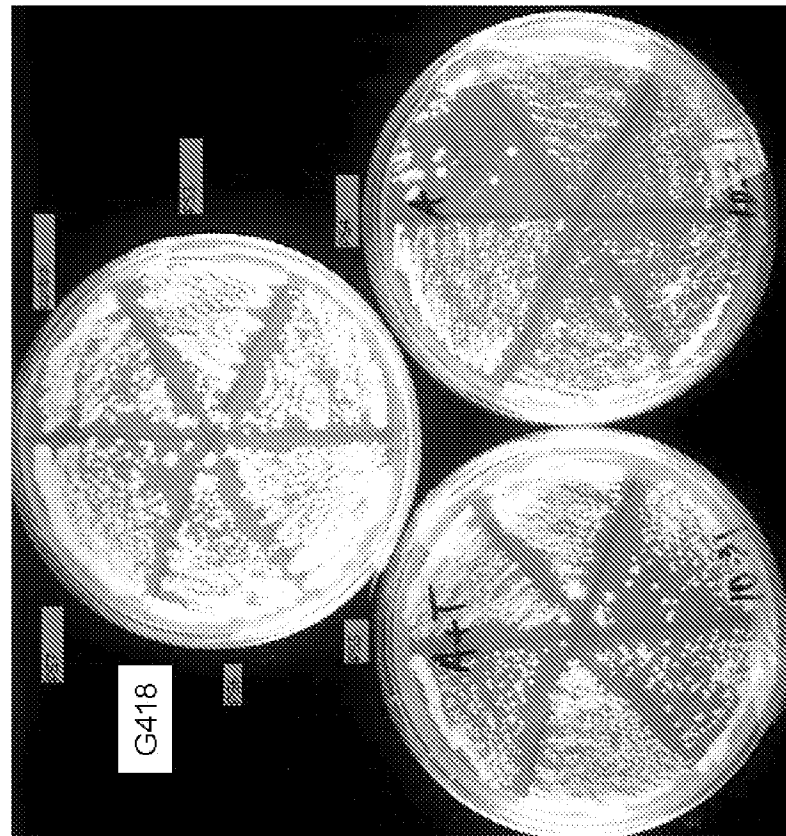


The run of six G's at codons 16 and 17 seems to be a hotspot for mutations

FIG. 26

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Isolation of weak suppressor plasmids



Rescreen of weak suppressor candidates. The large colonies on the A plate for the control are from G418^s cells which have lost the protease gene.

FIG. 27

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Weak suppression depends on plasmid

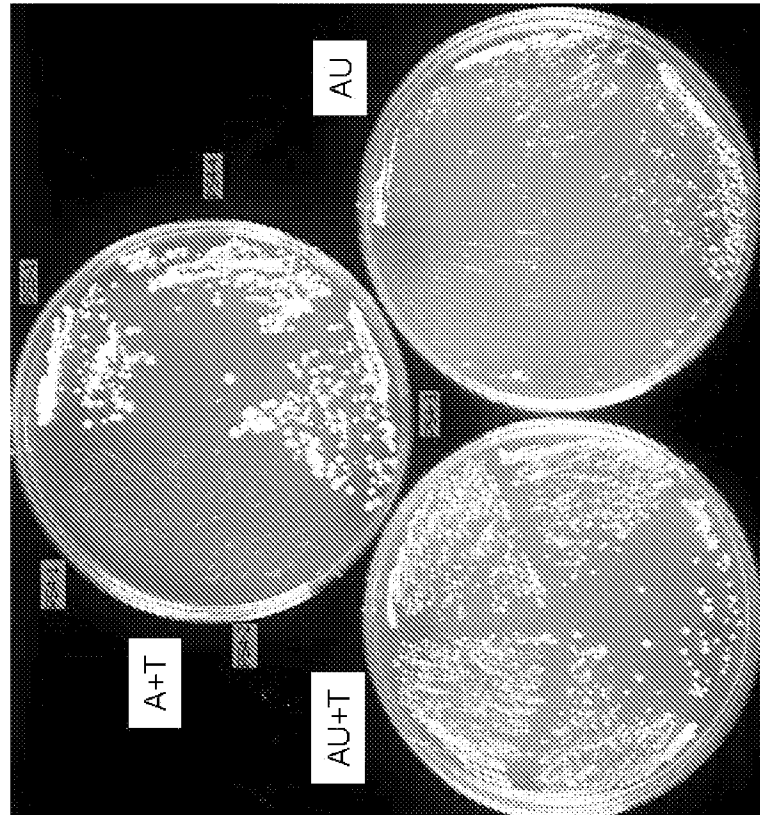


FIG. 28

For PS4, two colonies without plasmid do not grow on -T while two with plasmid do grow on -T.

After recovery in E. coli, one plasmid (pPS3-2) does not retest as suppressor while others do

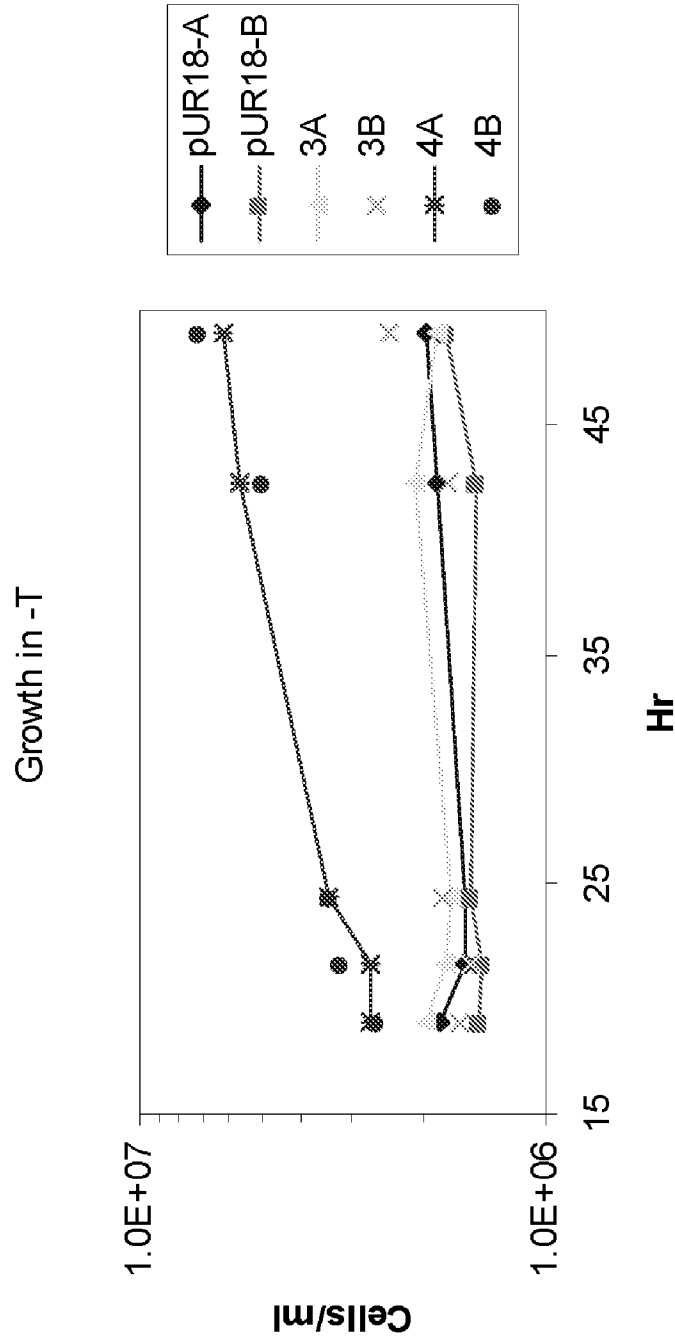


FIG. 29

Two transformants were analyzed for each plasmid, pUR18 (vector), pPS3-2 (false positive) and pPS4-10 (weak suppressor).

10 of 11 recovered plasmids retest
as weak suppressors by growth

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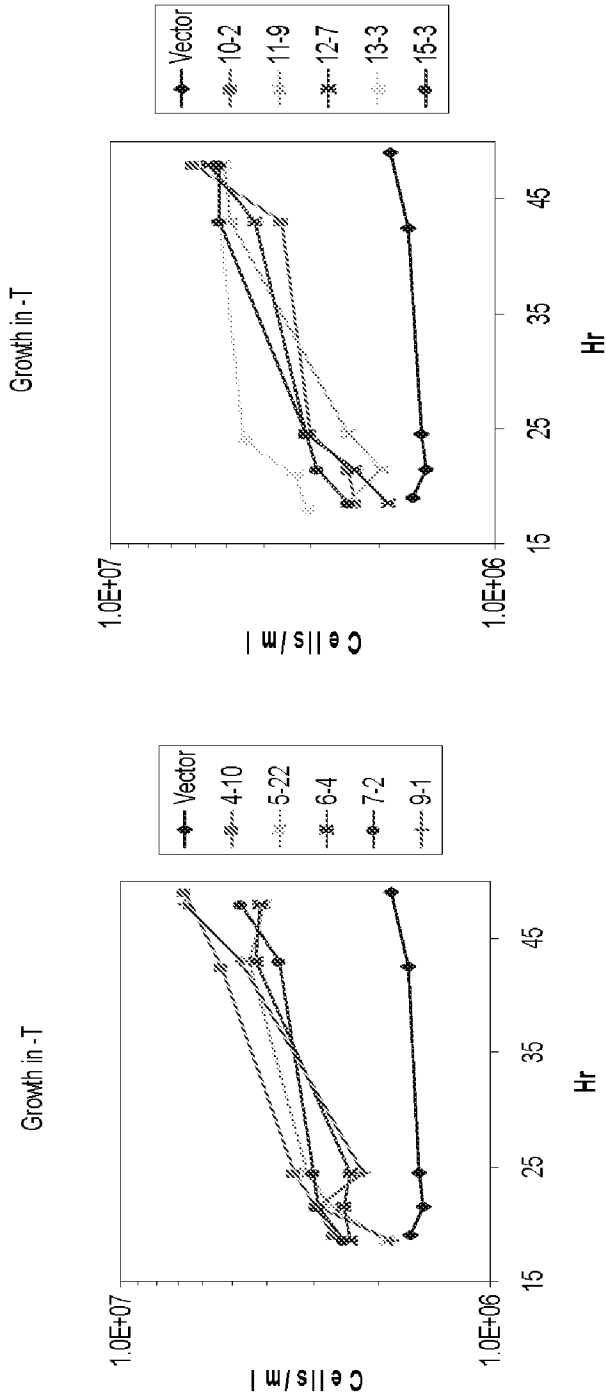


FIG. 30

10 of 11 recovered plasmids retest as weak suppressors by increased cell viability

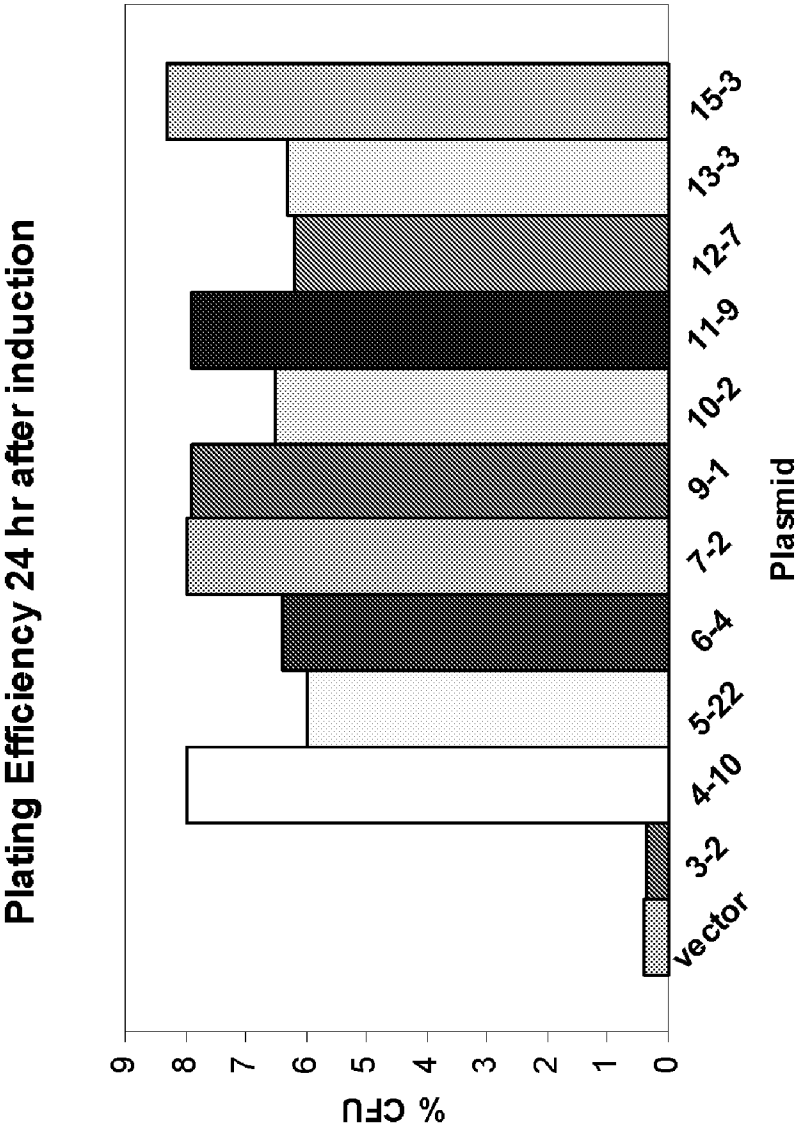
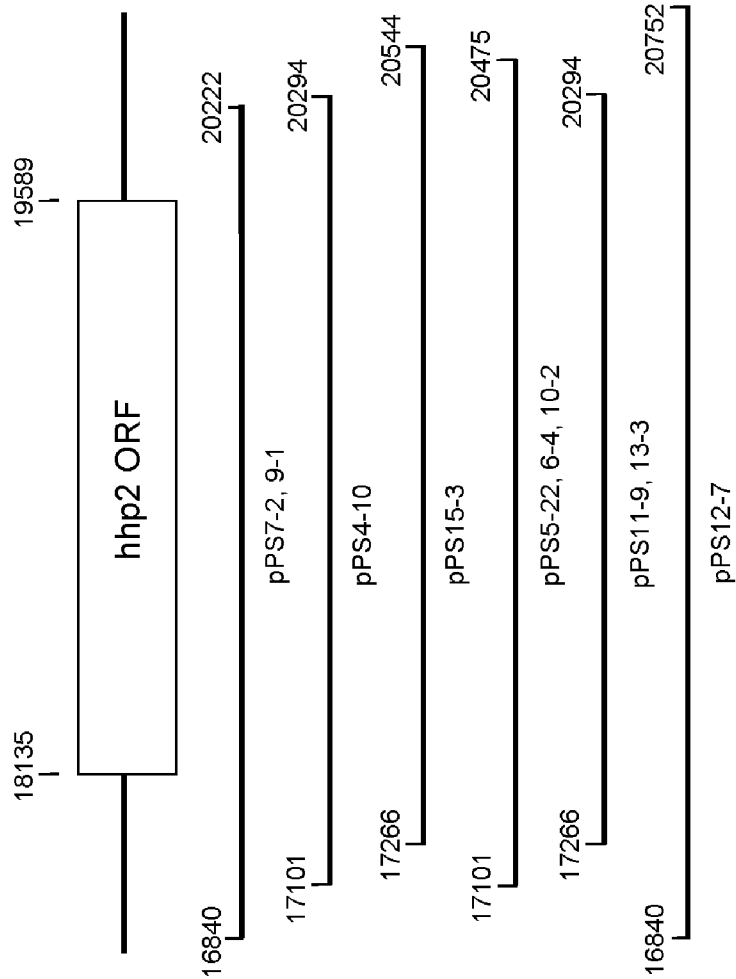


FIG. 31

The ten suppressor plasmids all contain the *hhp2* gene



The sequence numbering comes from cosmid SPAC23C4. None of the plasmids contain any other ORF. Six different plasmids were isolated from 4×10^4 transformants.

FIG. 32