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(54) **METHOD FOR SELECTIVE INACTIVATION OF VIRAL REPLICATION**

METHODE ZUR SELEKTIVEN INAKTIVIERUNG DER VIRALEN REPLICATION

PROCEDE D'INACTIVATION SELECTIVE DE REPLICATION VIRALE

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Description

[0001] This invention relates to methods uses and cells for screening for agents useful for treatment of viral infection.

Background of the Invention

[0002] A variety of agents are presently used to combat viral infection. These agents include interferon, which is a naturally-occurring protein having some efficacy in combat of certain selected viral diseases. In addition, agents such as AZT are used in the combat of an immunodeficiency disease, referred to commonly as AIDS, caused by the virus HIV-1.

[0003] Drug and Market Development, Vol 3. No. 9, pp. 174-180 (2/15/93), describes antiviral drug development. It states:

> The difficulties encountered in drug treatment of most infections pale when compared to viral infections. For example, it is at least theoretically (and often in practice) possible to attack a bacterium without harming the host. Unlike bacteria however, viruses replicate inside cells and utilize cellular machinery of the host for replication. As a result, development of antiviral therapeutics often represents a compromise between preferable killing, or at least arresting replication of, the virus, and not harming the host, or at worst, doing only minimal damage which can be justified by the potential gain.

[0004] It states that viral specific events can be targeted including:

* Virus attachment to cell membranes and penetration in cells;
* Virus uncoating;
* virus nucleic acid synthesis;
* Viral protein synthesis and maturation; and
* Assembly and release of infectious particles:

[0005] Specifically with regard to viral protein synthesis the authors state:

> In contrast to nucleic acid synthesis, viral protein synthesis utilizes host ribosomes (ribosomes are cell structures essential for translation of mRNA into protein) and mostly host-derived supplementary factors. As a result, protein synthesis inhibitors, in general, are as likely to exhibit host toxicity as they are to exert antiviral effects. Antisense oligonucleotides, however, may be of value in specifically inhibiting viral protein synthesis. Briefly, antisense oligonucleotides are short DNA fragments that are complementary to mRNA (sense strands) and can prevent mRNA-directed protein synthesis by binding to mRNA. RNA molecules have also been constructed to contain sequences complementary to those of sense DNA strands (and their corresponding mRNA). Although antisense constructs have been shown to inhibit viral protein synthesis in vitro, their effectiveness in vivo has not yet been conclusively demonstrated. Among others, current challenges for oligonucleotide therapeutics include delivery to virus-infected cells, the stability of such molecules in vivo and distribution throughout the body.
> Ribosome inactivators represent another approach for viral protein synthesis inhibition. GLQ223 (Genelabs; Redwood City, CA) is a ribosome inactivator undergoing clinical testing (GLQ223 is a purified preparation of trichosanthin (cucumber plant derivative)). A ribosome inactivator would interfere with cellular translation machinery, effectively preventing generation of new viral proteins.
> Sonenburg, 2 The New Biologist 402, 1990 describes virus host interactions at the level of initiation of translation and states that two initiation factors eIF-2 and eIF-4F play significant roles in a number of virus host interactions. He states "[a]n understanding of the mechanisms responsible for these virus-host interactions is of great signifigance for future therapeutic approaches to viral disease."

Summary of the Invention

[0006] The present invention relates to methods for screening for agents which are effective in inhibiting the translational system used by a virus during infection of a host cell. The screening method utilizes a protocol in which potentially useful agents are brought into contact with viral internal ribosomal entry site (IRES) nucleic acid sequences in order to determine whether those agents can specifically inhibit translation initiation. Once isolated, the viral specific agents can be formulated in therapeutic products (or even prophylactic products) in pharmaceutically acceptable formulations, and used for specific treatment of viral disease with little or no effect on uninfected virus host cells.

[0007] Specifically, in one aspect, applicant provides a screening method cells and the use of the cells as set out in the claims in which a target virus nucleic acid sequence or domain responsible for preferential translation of viral RNA

over host RNA is used in a selection protocol. While several specific examples of such viral nucleic acid sequences or domains are provided below in the form of IRES elements, 5'- untranslated regions containing specific viral sequences, and upstream open-reading frames containing such sequences, these are used only to exemplify a general method by which other virus nucleic acid sequences can be used in such protocols. Use of any one of these virus nucleic acid sequences within a cell translation system provides a means by which anti-viral agents can be discovered.

[0008] The claimed method does not include targeting of agents to viral sequences involved in frame shifting (which is not a target nucleic acid that is preferentially translated as defined herein), such as described by Dinman and Wickner, 66 J. Virol. 3669, 1992; Jacke et al., 331 Nature 280, 1988; Wilson et al., 55 Cell 1159, 1988; Inglis and Brierly, WO 90/14422; and Goodchild and Zamecnik, WO 87/07300.

[0009] Any agent which binds to such viral IRES nucleic acid and/or which causes a significant reduction in translation of viral message is potentially useful in the present invention. Such agents can be screened to ensure that they are specific to viral translation systems and have no effect on uninfected host cell translation systems such that the agent can be used in a therapeutic or prophylactic manner. If such agents have some effect on host cell systems they may still be useful in therapeutic treatment, particularly in those diseases which are life threatening, such as HIV-1 infection.

[0010] Such agents may interact either directly with the target viral nucleic acid, for example, by hybridization with the nucleic acid, e.g., antisense RNA or DNA, or may bind or interact with other components of the viral translation system (i.e., those host and/or viral components whether nucleic acid and/or protein which allow translation of viral mRNA to occur in vivo), such as proteins used by the virus to promote translation of its RNA, rather than host RNA involved in that system, e.g., antibodies. Additionally, agents may include any nucleic acid molecule which binds to viral or cellular components which otherwise would partake in preferential viral nucleic acid translation, but upon binding said nucleic acid molecule become unable to be preferentially translated. However, while antisense nucleic acid and antibodies may exemplify aspects of the present invention, applicant is particularly concerned with identification of agents of low molecular weight (less than 10,000, preferably less than 5,000, and most preferably less than 1,000), which can be more readily formulated as useful antiviral agents. Thus, in a preferred embodiment, the invention features such low molecular weight agents, and not antisense molecules or antibodies.

[0011] Thus, in a first aspect the invention features a method for screening for an antiviral agent. The method includes providing a target viral IRES nucleic acid sequence, translationally linked to a polynucleotide encoding a reporter polypeptide. The method then further includes contacting the target viral translation nucleic acid sequence with a potential antiviral agent under conditions which allow synthesis of the reporter polypeptide in the absence of the agent. The method finally includes determining whether the agent reduces the level of translation of the reporter polypeptide. Any agent which does reduce this level is potentially a useful antiviral agent.

[0012] Specifically, the method involves determining whether a potential agent interacts with a virus or cellular component which allows or prevents preferential translation of a virus RNA compared to a host RNA under virus infection conditions; and determining whether any interaction of the agent with the component reduces the level of translation of a RNA of the virus.

[0013] By "screening" is preferably meant a process in which a large number of potentially useful agents are processed in the method of this invention. It is generally a process distinct from a single experiment in which a single agent is studied in detail to determine its method of action.

[0014] The viral IRES nucleic acid sequence is exemplified by IRES elements which allow cap-independent translation of associated ribonucleic acid, and 5' untranslated regions of influenza virus RNA which allow preferential cap-dependant translation of associated RNA.

[0015] By preferential translation is meant that the RNA is translated at a higher rate or with higher yield of protein than host cell RNA under virus-infection conditions. In addition, the host cell RNA may be translated at a slower rate or with lower protein yield than in non-infected conditions. Such preferential translation en be readily detected as described below. In the case of most viruses, preferential expression of viral proteins means that synthesis of viral proteins represents at least 50% of total de novo protein synthesis, as may be detected, for example, by pulse-labeling experiments in viral-infected cells. In such cases, viral proteins may usually be distinguished as major bands when labeled proteins are separated by gel electrophoresis. In the case of retroviruses, preferential expression of viral proteins means that the level of viral proteins synthesized increases disproportionately beyond the level of viral RNA synthesized (Cullen, Cell 46: 973, 1986). Such a disproportionate increase can be detected by quantitating levels of viral RNA and protein synthesis in infected cells by, for example, Northern blotting and nuclease protection assays for RNA synthesis and immunoprecipitations and gel electrophoresis for labeled proteins.

[0016] By virus infection conditions is simply meant conditions within a host cell after infection with the target virus such that the viral translation system is operative. Such a viral translation system will usually include host cell proteins, nucleic acids and other components.

[0017] By reporter polypeptide is simply meant a peptide which is readily detectable, either by providing a colorimetric signal under certain environmental conditions or some other signal well known to those of ordinary skill in the art, as described below. The IRES elements afford preferential translation of viral mRNA over host cell mRNA when the host

cells are infected by the virus; and the virus from which that signal is selected is chosen from the picornavirus family, Hepatitis viruses A, B, and C, influenza virus, HIV, Herpes virus, and cytomegalovirus.

[0018] In more preferred embodiments, the method further includes determining whether an agent active in the above method has little or no effect on the translational machinery of an uninfected viral host cell, and further determining whether the agent is active under in vivo conditions. Such agents are then formulated in a pharmaceutically acceptable buffer.

[0019] By pharmaceutically acceptable buffer is meant any buffer which can be used in a pharmaceutical composition prepared for storage and subsequent administration, which comprise a pharmaceutically effective amount of an agent as described herein in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. Id. at 1449. In addition, antioxidants and suspending agents may be used. Id.

[0020] In some preferred embodiments, the method of this invention includes forming a protein translation mixture which includes (i) a viral mRNA construct, the mRNA construct comprising (a) an internal ribosome entry site (IRES) region and downstream of the IRES region, a first reporter protein coding region, (ii) ribosomes, and (iii) an agent to be tested, incubating the components of the translation mixture under conditions effective to produce from the first reporter protein coding region a reporter protein, and examining the mixture for the presence of reporter protein produced by such translation mixture, and the agent is a useful anti virus agent if the reporter protein produced in the presence of the test agent is less than an amount of reporter protein produced in the absence of the test agent.

[0021] Preferably, the IRES region is derived from a picornavirus IRES region sequence; the IRES sequence is selected from the group consisting of an enterovirus, rhinovirus, cardiovirus, and aphthovirus IRES sequence; the IRES region is selected from the group consisting of an hepatitis A virus IRES sequence, an hepatitis B virus sequence and an hepatitis C virus IRES sequence; the protein translation mixture is a cell-free extract; the 5'-end of the viral mRNA construct includes a eukaryotic mRNA 5'-terminal cap and untranslated region (UTR) and downstream of the cap and UTR region, a second reporter protein; and the translation mixture is contained in a cell.

[0022] In another example, the method includes forming a binding mixture comprising a cellular or viral translation initiation protein, an IRES element ribonucleotide sequence, and an agent to be tested, incubating the components of the binding mixture under conditions effective to bind the initiation protein to the IRES element, and examining the mixture for the presence of binding of the initiation protein to the IRES element. The agent is a useful antivirus agent if the extent of binding of the initiation protein to the IRES element is less than that observed in the absence of the agent.

[0023] Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of Figures

[0024]

Figure 1 shows the terminal stem, central domain, and apical stem loop of adenovirus VAI RNA (Ma, Y. and M. B. Mathews. 1993. Comparative analysis of the structure and function of adenovirus virus associated RNAs. J. Virol. 67:6605-6617).

Figure 2 shows the antisense VA (ava) oligodeoxynucleotide species ava 1, ava 2, ava 3 and ava 9 annealed to complementary sequences of VAI RNA.

Figure 3 shows the sequences of antisense species and complementary VAI RNA regions, i.e., VAI RNA. antisense oligodeoxynucleotides (ODN).

Figure 4 shows the result of *in vitro* translation assay. Column 1: (-) mRNA; column 2: (+) mRNA; column 3: (+) mRNA, (+) reovirus dsRNA; column 4: (+) mRNA, (+) reovirus dsRNA, (+) VAI RNA. Columns 5-9: (+) mRNA, (+) reovirus dsRNA, (+) VAI RNA, and antisense as follows: column 5: ava 1; column 6: ava 2; column 7: ava 3; column 8: ava 9; column 9: ava 15.

Figure 5 shows human rhinovirus 14 5'NTR sequence and predicted secondary structure (Le, S.-Y., and Zuker, M. (1990) J. Mol. Biol. 216, 729-741). The initiating AUG start codon for the polyprotein, at nucleotide ("nt") 625, is shown as a shaded box, non-initiating AUG codons are shown as clear boxes. The YnXmAUG motif found in all picornavirus IRES elements and the 21-base conserved sequence found in all rhinovirus and enterovirus IRES elements are underlined. Nucleotide positions on the rhinovirus genome are marked by numbers.

Figure 6 shows a schematic diagram of mRNAs used for *in vitro* translation studies. A) bCRL mRNA containing the β-globin 5' NTR driving translation of the CAT reporter gene, and rhinovirus IRES driving translation of the luciferase reporter gene. B) bL mRNA containing the β-globin 5' NTR driving translation of the luciferase reporter gene. Lines represent β-globin 5' non-translated region (NTR), rhinovirus IRES, or 3' NTRs, as indicated. Boxes represent

reporter genes CAT (chloramphenicol acetyl transferase) and luciferase.

Figure 7 shows *in vitro* translation of bLuc and bCRL mRNAs. Translation reactions were performed in duplicate as described by Lee, K. A. W., and Sonenberg, N. (1982) Proc. Natl. Acad. Sci. USA 79, 3447. Lane M, marker proteins; lanes 1-2, no mRNA; lanes 3-4, bL mRNA; lanes 5-6, bL mRNA with anti-IRES-oligo; lanes 7-8, bCRL mRNA; lanes 9-10, bCRL mRNA with anti-IRES-oligo. Bands corresponding to luciferase and CAT translation products are indicated, along with protein markers of 30, 46, and 69 kDa.

Figure 8 shows luciferase activity assay of bL and bCRL mRNA translation reactions in the absence and presence of antisense (anti-IRES-oligo) and control (control-oligo) deoxyoligonucleotides. Translation reactions and luciferase activity assays were performed as described in text. Relative light units from two independent replicates were averaged and luciferase activity from bL and bCRL translations normalized to 100 for comparison. Translation reactions contained: lane 1, no mRNA; lane 2, bL mRNA; lane 3, bL mRNA and anti-IRES-oligo; lane 4, bL mRNA and control-oligo; lane 5, bCRL mRNA; lane 6, bCRL mRNA and anti-IRES-oligo; lane 7, bCRL mRNA and control-oligo.

Description of the Preferred Embodiments Antiviral agents

[0025] Given the large number of drugs available for treating infections caused by more complex organisms such as bacteria, it is remarkable how few drugs are available for treating the relatively simple organisms known as viruses. Indeed, most viral diseases remain essentially untreatable. The major difficulty in developing anti-viral drugs is that, unlike bacteria, viruses replicate inside host cells and utilize the machinery of those cells for replication, sharing many nutritional requirements and synthetic pathways with their hosts. As a result, it is difficult to identify agents that kill or arrest replication of a virus without also harming the host. Even those anti-viral drugs that have been approved for use in humans often have side effects which limit their utility.

[0026] The majority of existing anti-viral drugs are nucleoside analogs or other agents that exert their effects through an enzyme involved in producing new copies of the viral genetic material, such as a nucleoside kinase or a polymerase or reverse transcriptase or replicase. These analogs are typically metabolized into nucleotide analogs that inhibit production of viral nucleic acid, for example by inhibiting a polymerase or by causing premature chain termination of growing viral nucleic acids. The efficacy of such drugs depends on two key factors. The first is that the target virus utilizes at least one virus-specific enzyme, encoded by the virus and used only by the virus, in the pathways which result in the copying of its genetic material. The second is that this enzyme is more sensitive to the drug or more efficient in utilizing it than any corresponding enzyme in the host. However, because viral and cellular nucleic acid metabolism are so similar, it is difficult to find anti-viral agents that are not used to some extent by host cell enzymes. This limits the dose of anti-viral drug that can be tolerated, which in turn may limit the utility of the drug.

[0027] Even in the case where a drug is tolerated at an effective dose, its effectiveness can be reduced markedly by the ability of a virus to mutate relatively rapidly, evolving new versions of the viral enzyme which do not utilize the drug as efficiently or which are less inhibited by the drug.

[0028] There is thus a clear need for novel anti-viral drugs that will be effective at doses tolerated by the host and that will be more difficult for viruses to evade by mutation.

[0029] The present invention provides novel methods and uses for discovering such drugs and for treating illnesses with the drugs discovered. The methods and uses of this invention are based in the observation that many viruses take over control of protein synthesis (translation of messenger RNA) in cells they infect. The viral proteins are synthesized preferentially over host proteins in infected cells. This preferential synthesis of viral proteins is important to the replication of the virus. Drugs which reduce or prevent the viral takeover of protein synthesis are therefore effective anti-viral agents.

[0030] Such drugs have significant advantages over current anti-viral agents. As noted above, the targets for the majority of the latter are enzymes involved in the synthesis of viral nucleic acids, and because host cells also contain enzymes active in the synthesis of nucleic acids it is difficult to hit the viral enzymes without also hitting the host ones. Similar problems are likely to occur for any drug target which is an active catalyst in the synthesis of a material required by both the virus and the host cell. In the methods of the present invention, these problems are avoided because the drug targets are not active catalysts in a synthetic pathway: they are devices used by a virus to secure preferential access to a synthetic pathway (protein synthesis), rather than catalysts in such a pathway. As weapons used by the virus in its attack on the host, these devices do not have any parallels within the host. Drugs which interfere with these devices therefore have minimal side effects on the host.

[0031] Such drugs are more effective than current drugs, for two reasons. First, their minimal side effects allow them to be used at higher doses. Second, it is possible for these drugs to be intrinsically more injurious to their targets than is tolerable for drugs whose targets have host homologues, because if the latter drugs are intrinsically too injurious they may harm the host homologues to some extent.

[0032] Viruses are also less able to evolve resistance to drugs which target viral translational hijacking devices. These devices must of necessity interact with host-cell components involved in protein synthesis, and the need to maintain

these interactions means that the virus is limited in the extent to which it can mutate its hijacking devices. If it mutates too far to avoid a drug, it may no longer be able to hijack protein synthesis. This limitation is particularly problematic for the virus because it may need to make larger changes to evade an hijack-blocking drug than to evade a drug whose target is a synthetic enzyme with a host homologue, because, as noted above, the hijack-blocking drug may be intrinsically more injurious to its target.

[0033] In summary, the present invention provides a means to discover and utilize novel anti-viral drugs with important advantages over current such drugs, namely fewer side effects and a reduced likelihood of the evolution of resistant viruses.

[0034] The methods of this invention are based in the observation that many viruses take control over the process of protein synthesis (translation of mRNA) in cells they infect. Viruses use a variety of mechanisms to effect this takeover, including but not limited to the use of special viral nucleic acid sequences which ensure preferential translation of viral RNAs (see e.g., Pelletier et al., Mol. Cell. Biol, 8, 1103-1112, 1988; Trono et al. Science 241, 445-448; Sonenberg & Meerovitch, 1990; Garfinkel & Katze, J. Biol. Chem. 267, 9383-9390, 1992), recruitment of cellular proteins to interact with these special sequences (see e.g., Jang SK & Wimmer E, Genes Dev. 4, 1560-1572, 1990), modification or degradation of host-cell components which participate in translation or its control (see e.g., Katze MG et al., J. Virology 62, 3710-3717, 1988, Lee et al., Proc. Natl. Acad. Sci. USA 87, 6208-6212, 1990), and disablement of cellular defenses mounted in response to the infection (see e.g., review by Katze MG, J. Interferon Res. 12, 241-248, 1992).

[0035] These methods are exemplified herein with descriptions of viral IRES nucleic acid sequences responsible for preferential translation of viral RNAs. The use of these examples is in no way intended to limit the scope of the invention.

Methods to screen potential agents

[0036] Methods to screen potential agents for their ability to disrupt or moderate viral effects on translation can be designed without detailed knowledge of the precise interaction between viral and cellular components, although such a knowledge can certainly be helpful. In principle, many of the numerous methods which have so far been described to identify viral and cellular components involved in effects on translation can be readily adapted to detect interference with the interaction between these components. Thus, for example, if it has been found that viral infection leads to the phosphorylation, dephosphorylation or other modification of a given component, or to a change in its catalytic activity such as the inhibition of that activity, or to enhanced synthesis or degradation of this component, or to any other observable effect described in the foregoing disclosure, then agents can be screened for their ability to prevent or moderate this effect on the component in question. The screening can be performed by adding the test agent to intact cells which have been infected by virus and then examining the component of interest by whatever procedure has been established to demonstrate the viral effect on this component. Purified or partially purified components which have been determined to interact with one another by the methods described above can be placed under conditions in which the interaction between them would normally occur, with and-without the addition of the test agent, and the procedures previously established to analyze the interaction can be used to assess the impact of the test agent. In this approach, the purified or partially purified components may be prepared by fractionation of extracts from uninfected and infected cells, or they may be obtained by expression of cloned genes or cDNAs or fragments thereof, optionally followed by purification of the expressed material.

[0037] The present invention is particularly concerned with screening methods and uses which measure the activity or expression of "reporter" protein, that is, an enzyme or other detectable or selectable protein, which has been placed under the control of the IRES nucleic acid sequence. Test methods may also be employed which have been configured such that the component(s) implicated in the viral effect controls the activity or expression of a "reporter" protein, that is, an enzyme or other detectable or selectable protein. In the case, for example, where a kinase has been implicated in the viral effect, the test method might be configured in such a way that phosphorylation of a particular protein by the kinase leads to the activation or inhibition of that protein or of some other protein controlled by that protein. In yeast, for example, phosphorylation of eIF2-α by the GCN2 protein (or by mammalian p68 kinase substituting for GCN2) leads to an inhibition of the initiation of translation, which in turn leads to an increase in the synthesis of the GCN4 protein, which in turn induces the synthesis of further proteins involved in amino acid biosynthesis. "Reporter" proteins can be readily fused to the GCN4 protein at the genetic level so that the synthesis of these reporters is effectively induced by the initial phosphorylation event catalyzed by GCN2 or mammalian p68.

[0038] Similar approaches can be used to detect modulation by test agents of the activity of a variety of other components which might be implicated in viral effects on translation. The effect of a test agent on a protease, for example, can be monitored by following the survival in an *in vitro* reaction of a reporter protein which is a target for that protease. Similarly, the effect of a test agent on a nuclease can be monitored by following the appearance in an *in vitro* translation reaction or *in vitro* transcription-translation reaction of a reporter protein translated from a suitably configured coding sequence provided to the reaction.

[0039] Proteins suitable for use as reporters in such assays include, but are not limited to, easily assayed enzymes

such as β-galactosidase, luciferase, β-glucuronidase, chloramphenicol acetyl transferase, and secreted embryonic alkaline phosphatase; proteins for which immunoassays are readily available such as hormones and cytokines; proteins which confer a selective growth advantage on cells such as adenosine deaminase, aminoglycoside phosphotransferase (the product of the neo gene), dihydrofolate reductase, hygromycin-B-phosphotransferase, thymidine kinase (when used with HAT medium), xanthine-guanine phosphoribosyltransferase (XGPRT), and proteins which provide a biosynthetic capability missing from an auxotroph; proteins which confer a growth disadvantage on cells, for example enzymes that convert non-toxic substrates to toxic products such as thymidine kinase (when used with medium containing bromodeoxyuridine) and orotidine-5'-phosphate decarboxylase (when used with 5-fluoroorotic acid); and proteins which are toxic such as ricin, cholera toxin or diphtheria toxin.

[0040] While many of the methods so far described for selecting test agents have involved examining the impact of these agents on the interaction between two or more components in *in vitro* reactions, the present invention is directed to methods, cell and uses in which the interacting components are brought into contact with one another within cells rather than in in *vitro* reactions. In this approach, coding sequence(s) encoding part or all of a component or components would be introduced into a selected type of cell. Coding sequences for this approach include cloned genes or cDNAs or fragments of either or fragments amplified by the polymerase chain reaction or natural RNAs or transcribed RNAs or the like. Several variations of the approach are possible. In one variation, a coding sequence is introduced for a first component into a cell known to contain components with which this first component will interact. Thus, for example, a coding sequence for a viral component is introduced into a cell which is a normal target for infection by the virus in question. Agents are tested to select those which block the effect of the viral component within the cell into which the coding sequence has been introduced. In another variation, coding sequences for two or more components which interact with one another might be introduced into a cell, and agents tested for their ability to moderate the interaction between these components, this interaction being followed by the procedures previously established as suitable for the purpose. The cell into which the coding sequences are introduced can be one which would normally be a target for infection by the virus in question. Alternatively and usefully, the cell can be one which is easier to grow, manipulate and test such as a yeast cell. Indeed, there are distinct advantages to reconstructing a translation control mechanism in heterologous cells, in which the interactions between the components involved are easier to study than they are when those components are in their normal environment. In the case of yeast, in particular, the powerful genetic approaches available often make it possible to identify and isolate the yeast homologues of genes from higher eukaryotes more quickly than the corresponding genes can be identified in the higher eukaryotes.

[0041] From the foregoing it should be apparent that one skilled in the art is able to choose from a wide variety of methods at each stage in the identification of components involved in viral effects on translation, in the characterization of the interaction between these components, and in the implementation of screening tests to select compounds which moderate or abolish the interaction between these components.

Nucleic acid targets

[0042] One particularly useful macromolecule target is an IRES nucleic acid sequence. There now follows a detailed review of useful methods of this invention which are based upon targeting agents of this invention to such nucleic acids.

[0043] Viruses are believed by Applicant to employ nucleic acid sequences responsible for preferential translation of viral RNAs. Viruses whose RNAs are believed to be preferentially translated because of specific viral nucleic acid sequences currently include picornaviruses, hepatitis B virus, hepatitis C virus, influenza virus, adenovirus and cytomegalovirus.

[0044] Picornaviruses are an important class of viruses responsible for a broad array of human and animal diseases (reviewed in Chapters 20-23 in Fields BN, Knipe DM (eds): Fields Virology, ed. 2, Raven Press, New York, 1990). They include polioviruses, rhinoviruses (the most frequent cause of respiratory tract infections), coxsackie viruses (a cause of gastrointestinal illnesses, myocarditis and meningitis), hepatitis A virus, and foot-and-mouth disease viruses. Picornaviruses are single-stranded RNA viruses whose RNA genomes are positive-sense and nonsegmented. The genomic RNA strand inside each virus is translated when the virus enters a host cell. One of the proteins translated from the incoming RNA genome is an RNA-dependent RNA polymerase which copies the viral genome to produce additional full-length viral RNAs. Some of these RNAs are translated to produce additional viral proteins, and some are packaged as RNA genomes into a new generation of viruses. Each RNA is translated into a single "polyprotein" which is cleaved as it is translated to yield individual viral proteins.

[0045] One of the early effects of infection with a picornavirus is a shutoff of host protein synthesis. At least in the case of poliovirus infection, this appears to be due to cleavage of a host cell protein known as p220, one of three polypeptide constituents of the initiation factor eIF-4F, also known as cap-binding protein complex. eIF-4F is required for initiation of protein synthesis from host cell mRNAs, which bear a structure known as a cap at their 5'-ends. eIF-4F is believed to bind to the cap structure and participate in the unwinding of secondary structure adjacent to the cap in the 5'-untranslated leader (5-UTR) of mRNAs. This unwinding is necessary for ribosomes to bind to the mRNA and migrate

along it to the AUG codon which represents the start of the coding sequence. Thus, by cleaving one of the subunits of eIF-4F, picornaviruses prevent cap-dependent initiation of translation of host-cell mRNAs, and thereby disable host-cell protein synthesis. Viral RNAs can be translated, however, because they utilize a cap-independent mechanism for initiation; indeed, picornaviral RNAs do not have caps at their 5'-ends. Some but not all scientists in the field believe that the cap-independent mechanism involves sequences within the 5'-UTR of the viral RNAs known as internal ribosomal entry sites (IRES, or IRES elements) or ribosomal landing pads (RLPs) (reviewed in Sonenberg & Meerovitch, 1990). As their names imply, these are sequences which enable ribosomes to bind to viral RNAs at internal sites rather than at the 5'-ends of these RNAs; having bound, the ribosomes can then migrate to the AUG initiator codon and begin translation. Such binding at internal sites allows the ribosomes to bypass the virus-induced defect in the normal cap-dependent mechanism of initiation.

[0046] The existence of IRES. elements in picornaviral RNAs was inferred from several different types of observation (see Sonenberg & Meerovitch, 1990). So, for example, viruses with mutations in the 5'-UTR were found to make significant amounts of viral RNA but very little viral protein. More direct evidence came from the studies with dicistronic mRNAs in which the poliovirus 5'-UTR (for example) was positioned between the coding sequences for two separate proteins in a single mRNA. Experiments both *in vivo* and *in vitro* demonstrated that the second cistron could be translated under conditions in which the first was not, for example, in virus-infected cells or in the presence of an inhibitor of cap-dependent translation, but that in the absence of the viral 5'-UTR from the intercistronic space, translation of the second cistron depended on translation from the first. Further refinement of such experiments, involving for example progressive deletions from either end of the 5'-UTR, permitted more precise definition of the region within the 5'-UTR which constitutes the IRES element. Proteins which interact with IRES elements were then identified by gel-retardation assays and UV-cross-linking studies.

[0047] Evidence that IRES elements are indeed important for translation has been obtained by demonstrating that the 5'-UTR of encephalomyocarditis virus (EMCV) or fragments thereof can act as competitive inhibitors of translation *in vitro* (Pestova et al. (1991) J. Virol, 6194-6204) and that short DNAs complementary to the EMCV IRES element can also block translation in vitro. (Shih et al., (1987) J. Virol. 2033-2037, Pestova et al. (1989) Virus Research, 107-118 Borovjagin et al., (1991) Nucl. Acids Res., 4999-5005).

[0048] Despite these studies there is still controversy about whether translational initiation at IRES elements really occurs, and some evidence to suggest that it does not. Thus, one authority in the field has argued strongly that important controls were omitted from crucial experiments supporting the existence of IRES elements, characterizing these experiments as flawed or inconclusive and IRES elements as artifacts (Kozak (1989) J. Cell Biol. 229-241; Kozak (1992) Crit. Rev. Biochem. Mol. Biol. 385-402). It has also been demonstrated that if a cap is added to poliovirus RNA, which does not normally have such a structure, translation of the poliovirus RNA is inhibited (Hambridge SJ & Sarnow P, (1991) J. Virology 65, 6312-6315). This observation is at odds with the purported ability of ribosomes to initiate translation of poliovirus RNA by binding to IRES elements downstream of the 5'-cap.

[0049] Even if IRES elements do function as their proponents claim, the mechanism may not be unique to viruses. Thus it has been reported that internal ribosome entry sites exist within cellular mRNAs (Macejak & Sarnow (1991) Nature, 90-94; Jackson (1991) Nature, 14-15). The existence of such sites within cellular mRNAs would suggest that it may be difficult to identify compounds which prevent translational initiation at viral IRES elements without adversely affecting the translation of at least some cellular mRNAs.

[0050] Picornaviruses may not be the only viruses which utilize special sequences to enable ribosomes to bind at internal sites within RNAs and thus ensure preferential translation of viral proteins. Evidence for a similar mechanism has also been found in the case of hepatitis B virus and hepatitis C virus. Note that since hepatitis A virus is a picornavirus, this means that virtually all clinically significant hepatitis disease is caused by viruses which utilize internal ribosome entry sites.

[0051] Hepatitis B virus is a hepatovirus which can cause severe liver disease and which is very widespread (reviewed in chapter 78 of Fields BN, Knipe DM (eds): Fields Virology, ed. 2, Raven Press, New York, 1990). The virus has a very unusual genome and an equally unusual method of replication. In brief, the viral genome consists of partially double-stranded DNA. The negative-sense strand is a full circle, but the two ends of this circle are not covalently joined. The positive-sense strand is incomplete and its length is not the same in all molecules, so that the single-stranded region of the genome varies in length from approximately 15%-60% of the circle length in different molecules. When the virus infects a cell, the infecting genome appears to be converted to closed circular (cc) viral DNA which can be detected in the cell nucleus. This DNA is transcribed into (positive-sense) viral mRNAs, one of which encodes a reverse transcriptase which makes negative-sense DNA copies of viral RNA to produce further viral genomes. The (incomplete) positive-sense DNA strand of the genome is produced by partial copying of the negative-sense strand, with synthesis primed by a short viral oligoribonucleotide. The viral reverse transcriptase (P protein) is encoded within a long mRNA which also includes the coding sequence for the major viral core protein (C protein). The C-protein sequence is upstream of the P-protein sequence in the mRNA and partially overlaps it, in a different reading frame. Data from gene fusions which place a reporter gene downstream of the C-P overlap region suggest that translation of the P protein involves initiation at an

internal ribosome entry site within the C-protein coding sequence (Chang et al., (1990), Proc. Natl. Acad. Sci. USA 87, 5158-5162). This interpretation is supported by the observation that defined fragments of the C-protein sequence increase translation of the downstream cistron when placed between the two cistrons of a dicistronic mRNA or in the 5'-UTR of a monocistronic mRNA (Jean-Jean et al., (1989) J. Virology 63, 5451-5454). Thus, the ability to translate a crucial viral protein is highly dependent upon the presence of a specific viral nucleic acid sequence translationally linked to the coding sequence.

[0052] Hepatitis C virus also appears to utilize specific viral nucleic acid sequences to bypass the normal cellular method for initiation of translation. As its name implies, hepatitis C is a causative agent of the diseases formerly known as non-A, non-B hepatitis. Like picornaviruses it has a positive-sense, single-strand genome which is translated as a single open-reading frame, presumably into a polyprotein precursor which is then cleaved to provide mature viral proteins. Given the much more recent discovery of hepatitis C virus, much less is known about it than the picornaviruses, and the evidence supporting its use of IRES-like elements is unclear. Thus on the one hand, experiments based on *in vitro* translation reactions led to the conclusion that translation of viral RNAs can be initiated at internal ribosome entry sites, but on the other hand, experiments *in vivo* found no evidence for such a mechanism of initiation (Yoo *et al.* (1992) Virology 889-899).

[0053] Influenza viruses also cause a dramatic inhibition of host cell protein synthesis during infection, while viral proteins are synthesized selectively and efficiently. Influenza viruses are of course the etiologic agents of the eponymous disease (for a review of these viruses see chapters 39 & 40 of Fields BN, Knipe DM (eds): Fields Virology, ed. 2, Raven Press, New York, 1990). They too have single-stranded RNA genomes, but in their case the genome consists of negative-sense RNA and each gene exists on a separate RNA segment which is encapsidated separately into the virion; the viruses are thus of the type known collectively as segmented negative-strand RNA viruses. After infection the separate RNAs are copied into positive-sense RNAs for translation. This copying is catalyzed by a virus-coded RNA-dependent RNA polymerase protein, but requires short capped pieces from the 5'-ends of cellular mRNAs to act as primers. These primers are derived from cellular mRNAs through the action of a virus-encoded endoribonuclease. Thus, the first 10-13 nucleotides of each positive-sense, translatable, influenza viral RNA is derived from cellular mRNA.

[0054] In cells infected with an influenza virus, newly synthesized cellular mRNAs do not reach the cytoplasm (Katze & Krug, (1984) Mol. Cell. Biol. 4, 2198-2206), and translation of pre-existing mRNAs is blocked at both the initiation and elongation stages (Katze et al.,(1986) J. Virology 60, 1027). Evidence that specific RNA sequences in influenza virus mRNA ensure its preferential translation came from the fact that influenza mRNAs were selectively translated in cells infected by another virus, adenovirus, despite the shutdown of host protein synthesis in these cells (Katze *et al.* 1986). Further progress in understanding the preferential translation of influenza RNAs came with the development of a transfection-infection assay (Garfinkel & Katze, (1992) J. Biol. Chem. 267, 9383-9390). This was used to show that an exogenously introduced influenza viral gene was not subjected to the same translational blocks in infected cells as an exogenously introduced cellular gene. It was also concluded that translation of influenza mRNAs occurs in a cap-dependent manner, because such translation was inhibited by poliovirus infection, which blocks cap-dependent translation. Given that the 5'-ends of viral mRNAs are capped and derived from cellular mRNAs, this is not unexpected. For the same reason, it would not be expected that the 5'-UTR would play an important role in the preferential translation of influenza mRNA. Indeed, it was observed that there is nothing remarkable about the primary/secondary structure or length of the influenza 5'-UTR used for the transfection-infection assays described above. Unexpectedly, however, it has now been demonstrated that preferential translation of influenza mRNAs does depend on the 5'-UTR, and that the selectivity-determining region is surprisingly small, as small as 12 nucleotides. For comparison, a typical IRES element in a picornavirus has a length of about 400 nucleotides.

[0055] Most of the viruses so far described have been RNA viruses, but DNA viruses also appear to utilize special nucleic acid sequences which mediate preferential translation of viral RNAs. Adenovirus is an example of such a DNA virus (reviewed in chapters 60 & 61 of Fields BN, Knipe DM (eds): Fields Virology, ed. 2, Raven Press, New York, 1990). Adenovirus is responsible for various disorders including respiratory tract infections, conjunctivitis, hemorrhagic cystitis and gastroenteritis. The replicative cycle of adenovirus is significantly more complicated than that of the smaller picornaviruses and influenza viruses. Viral RNAs are transcribed from viral DNA by the host RNA polymerase II in two main phases, early and late transcription, with the late stage by definition starting with the onset of viral DNA synthesis, which is usually 6-9 hours after infection. That there is preferential translation of viral RNAs is demonstrated by a variety of observations. Host-cell protein synthesis is dramatically reduced in infected cells, even though cellular mRMA synthesis continues and there is no rapid breakdown of existing cellular mRNAs. Early in.infection, early viral mRNA constitutes less than 0.1% of the total mRNA in the cell, but 5-18% of the mRNA in polysomes, that is, 5-18% of the mRNA which is being actively translated.

[0056] The mechanisms by which adenovirus accomplishes its takeover of protein synthesis are not fully understood, but it has been demonstrated that dephosphorylation of a component of the cap-binding protein complex, eIF-4E, may play a role in this takeover (Huang & Schneider, (1991), Cell 65, 271-280). In support of this, it has also been shown that adenovirus mRNAs containing special sequences known as tripartite leader sequences are translated in a cap-

independent manner (Dolph et al., (1988) J. Virology 62, 2059-2066). Thus, preferential translation of adenovirus mRNAs also appears to depend upon specific viral nucleic acid sequences.

[0057] A DNA virus belonging to the herpes family, cytomegalovirus, may also utilize specific viral nucleic acid sequences to ensure preferential translation of viral RNAs. Cytomegalovirus is endemic in many populations, but many infections are subclinical in normal healthy individuals (reviewed in chapter 69 of Fields BN, Knipe DM (eds): Fields Virology, ed. 2, Raven Press, New York, 1990). The virus can cause serious illness, however, in immunosuppressed individuals, and has become a significant pathogen in recent years as a result of the rapid growth in the number of such individuals, some of them transplant recipients on immunosuppressive regimens, many of them sufferers from AIDS.

[0058] As viruses go, cytomegalovirus has a very large genome, and its replicative cycle and interactions with host cells are complex. Several observations suggest an important role for translational control of the production of important viral proteins (Geballe AP & Mocarski ES (1988), J. Virology.62, 3334-3340; Biegalke B & Geballe AP (1990) Virology 177, 657-667; Schleiss et al., (1991), J. Virology 65, 6782-6789). Thus, several cytomegalovirus proteins, including the glycoprotein gp48, are not synthesized efficiently until late in infection, although their mRNAs accumulate at earlier stages. Further investigations revealed an unusual *cis*-acting sequence in the 5'-UTR of gp48 that inhibits downstream translation in transfection assays and may mediate regulation of gp48 translation during infection, possibly be delaying such translation until conditions for it are most favorable. An essential element of the cis-acting sequence is an upstream open-reading frame in the 5'-UTR, that is, a' short coding sequence beginning with an AUG that is not the initiator AUG for the gp48 protein. Further evidence suggests that a cellular factor may be activated during cytomegalovirus infection and alleviate the inhibitory effects of the upstream open-reading frame. The latter may thus represent another viral nucleic acid sequence which at the correct stage of the viral replicative cycle is responsible for preferential translation of a viral RNA.

Specific viral nucleic acid sequences mediating preferential translation

[0059] Various approaches are available to determine whether specific viral IRES nucleic acid sequences are responsible for the preferential translation of viral RNAS. These include, but are not limited to, studies with chimeric RNAs having a detectable reporter polypeptide translationally linked to a viral nucleic acid sequence potentially responsible for the preferential translation; studies of naturally occurring and laboratory mutants of viral nucleic acid sequences; and transfection-infection assays.

[0060] A fruitful initial approach is often to construct chimeric RNAs having the coding sequence for a detectable reporter polypeptide linked to a viral nucleic acid sequence potentially responsible for the preferential translation of viral RNAs. Production of the detectable reporter polypeptide is then examined in translation conditions under which this reporter will not be produced unless the viral nucleic acid sequences ensure its translation. As a control, production of the detectable reporter polypeptide will also be examined under the same translation conditions from parallel constructs in which the reporter is not linked to the viral nucleic acid sequences under test. As an additional control, the chimeric RNA, or alternatively a second RNA added to each test, may include the coding sequence for a second detectable reporter polypeptide distinguishable from the first and translationally linked to RNA sequences responsible for ensuring normal translation of cellular mRNAs.

[0061] In some cases the translation conditions used for the test will be the translation conditions present in infected cells. In such cases the test can be performed by introducing the chimeric RNA or a DNA sequence encoding it into cells which previously, concurrently or subsequently are also infected with the virus under study. The transfection-infection assay described in more detail below is an example of such a test. As an alternative to performing the test in intact cells, the translation conditions present in infected cells can be reproduced *in vitro* by preparing extracts from infected cells and adding these to, or using them for, *in vitro* translations of the chimeric RNAs.

[0062] In other cases it may not be necessary to work with infected cells or extracts made from them. In some cases the chimeric RNA can be constructed in such as way that there will be limited or no production of the detectable reporter polypeptide in uninfected cells or *in vitro* translation extracts from such cells unless the test sequence linked to the coding sequence for the reporter allows preferential translation of the reporter. An example would be a chimeric RNA in which production of the detectable reporter polypeptide requires initiation of translation at an internal site within the RNA. In other cases it may be possible to add an inhibitor to uninfected cells or extracts made from them which blocks a step or pathway normally blocked during viral infection. An example would be the addition of cap analogs to inhibit cap-dependent initiation of translation.

[0063] Detectable reporter polypeptides suitable for use in chimeric RNAs or control RNAs include, but are not limited to, easily assayed enzymes such as β-galactosidase, luciferase, β-glucuronidase, chloramphenicol acetyl transferase, and secreted embryonic alkaline phosphatase; proteins for which immunoassays are readily available such as hormones and cytokines; proteins which confer a selective growth advantage on cells such as adenosine deaminase, aminoglycoside phosphotransferase (the product of the neo gene), dihydrofolate reductase, hygromycin-B-phosphotransferase, thymidine kinase (when used with HAT medium), xanthine-guanine phosphoribosyltransferase (XGPRT), and proteins

which provide a biosynthetic capability missing from an auxotroph; proteins which confer a growth disadvantage on cells, for example enzymes that convert non-toxic substrates to toxic products such as thymidine kinase (when used with medium containing bromodeoxyuridine) and orotidine-5'-phosphate decarboxylase (when used with 5-fluoroorotic acid); and proteins which are toxic such as ricin, cholera toxin or diphtheria toxin.

[0064] Viral nucleic acid sequences responsible for preferential translation of viral RNAs can also be identified by studies of naturally occurring and laboratory mutants. The latter may be constructed by a variety of procedures known to those skilled in the art, including but not limited to chemical treatment with mutagens, and the use of molecular biology techniques to generate insertions, substitutions, deletions and point mutations in viral nucleic acid sequences. The impact of various mutations on the preferential translation of viral proteins can then be assessed by the methods described above for studying such preferential translation.

[0065] In a related approach, the mutational analysis can be performed on viral nucleic acid sequences that are translationally linked to coding sequences for detectable reporter polypeptides within chimeric RNAs of the type described above. The impact of mutations within the viral nucleic acid sequences can be assessed by examining the production of the detectable reporter polypeptide under translation conditions which require a functioning viral nucleic acid sequence for the reporter to be synthesized. This approach can be particularly productive for detailed mapping and characterization of the regions within a viral nucleic acid sequence which are important for its function in ensuring preferential translation of viral RNAs.

[0066] Transfection-infection assays are another tool which can be used to identify viral nucleic acid sequences which ensure preferential translation of viral RNAs. As explained above, such assays involve the introduction into a cell by transfection of a gene or complementary DNA (cDNA) which encodes a reporter protein that can be assayed or detected, and infection of this cell with the virus under study. To use this type of assay to identify a viral nucleic acid sequence conferring preferential translation, different chimeric constructs would be made with the same reporter gene/protein. In some constructs the RNAs transcribed from this gene will contain normal cellular translation sequences, and in others they would contain viral nucleic acid sequences believed to be responsible for preferential translation of viral RNAs. If production of the reporter protein in infected cells is lower from RNAS containing cellular translation sequences than it is from RNAs containing viral nucleic acid sequences, this indicates that the viral sequences in question are capable of mediating preferential translation.

[0067] It will be evident to one skilled in the art that this type of transfection-infection assay can also be used to analyze mutations made in viral nucleic acid sequences in order to map and characterize the precise regions of these sequences responsible for mediating preferential translation.

[0068] 5'-untranslated leader sequences potentially containing sequence elements useful in the practice of this invention are known for a number of viruses and viral strains, as detailed in the following publications:

Coxsackievirus

[0069]

Jenkins O., 1987, J. Gen. Virol 68, 1835-1848
Ilzuka et al., Virology 156, 64.
Hughes et al., 1989, J. Gen. Virol. 70, 2943-2952.
Chang et al., 1989, J. Gen. Virol. 70, 3269-3280.
Chang et al., 1989, J, Gen. Virol. 70, 3269-3280.
Lindberg et al., 1987 Virology 156, 50.
Tracy et al., 1985 Virus Res. 3, 263-270.

Hepatitis A virus

[0070]

Cohen JI et al., 1987 Proc. Natl. Acad. Sci. USA 84, 2497-2501.
Paul et al., 1987, Virus Res. 8, 153-171.
Cohen et al., 1987, J. Virol. 61, 50-59.
Linemeyer et al.,1985 J. Virol. 54, 252.
Najariah et al., 1985 Proc. Natl. Acad. Sci. USA 82, 2627
Baroudy BM et al., 1985 Proc. Natl. Acad. Sci. USA 82, 2143-2147.

Poliovirus

[0071]

Racaniello & Baltimore 1981 Proc. Natl. Acad. Sci. USA 78, 4887-4891;
Stanway G et al.,1984 Proc. Natl. Acad. Sci. USA 81, 1539-1543.
La Monica N et al., 1986 J. Virology 57, 515.
Hughes PJ et al., 1986 J. Gen. Virol. 67, 2093-2102.
Hughes PJ et al., 1988 J. Gen. Virol. 69, 49-58.
Ryan MD et al.,1990 J. Gen. Virol 71, 2291-2299.
Pollard et al., 1989, J. Virol.,63, 4949-4951.
Nomoto et al., 1982 Proc. Natl. Acad. Sci. USA 79, 5793-5797.
Toyoda et al., 1984, J. Mol. Biol. 174, 561-585.

Rhinovirus

[0072]

Deuchler et al., 1987 Proc. Natl. Acad. Sci. USA 84, 2605-2609.
G. Leckie, Ph.D. thesis University of Reading, UK.
Skern T et al., 1985, Nucleic Acids Res. 13, 2111.
Callahan P et al., 1985 Proc. Natl. Acad. Sci. USA 82, 732-736.
Stanway et al., 1984 Nucl. Acids Res. 12, 7859-7875.

Bovine enterovirus

[0073]

Earle et al., 1988, J. Gen. Virol. 69, 253-263.
Foot-and mouth disease virus
Forss et al., 1984, Nucleic Acids Res. 12, 6587.
Beck et al., 1983, Nucleic Acids Res. 11, 7873-7885.
Villanueva et al., 1983, Gene 23, 185-194.
Beck et al., 1983, Nucleic Acids Res. 11, 7873-7885.
Carroll AR et al., 1984 Clarke Nucleic Acids Res. 12, 2461.
Boothroyd et al., 1982, Gene 17, 153-161.
Boothroyd et al., 1981 Nature, 290, 800-802.
Robertson et al., 1985, J. Virol. 54, 651.
Wendell et al., 1985 Proc. Natl. Acad. Sci. USA 82, 2618-2622.

Enterovirus type 70

[0074]

Ryan, MD et al. 1989 J. Gen. Virol.

Theiler's murine encephalomyelitis virus

[0075]

Ohara et al., 1988, Virology164, 245.
Peaver et al., 1988, Virology 165, 1.
Peayer et al., 1987, J. Virol. 61, 1507.

Encephalomyocarditis virus.

[0076]

Palmenberg et al., 1984 Nucl. Acids Res. 12, 2969-2985.
Bae et al., 1989 Virology 170, 282-287.

Hepatitis C virus

[0077]

Inchauspe et al., 1991 Proc. Natl. Acad. Sci. USA 88, 10293.
Okamoto *et al.,* 1992, v 188, 331-341
Kato et al., 1990, Proc. Natl. Acad. Sci. USA 87, 9524-9528
Takamizawa et al., 1991, J. Virology 65, 1105-1113
Okamoto et al., 1991, J. Gen. Virol 72, 2697-2704
Choo et al., 1991, Proc. Natl. Acad. Sci. USA 88, 2451-2455
Han et al., 1991 Proc. Natl. Acad. Sci. USA 88, 1711-1715

Influenza virus

[0078]

Fiers w et al., 1981, , J. Supramol Struct Cell Biochem (Suppl 5), 357.

[0079] The sequence of the 5'-UTR is AGCAAAAGCAGGGUAGAUAAUCACUCACUGAGUGACAUCAAAAUC. The 12 nucleotides underlined are conserved in all influenza mRNAs.

[0080] Also known is the sequence of hepatitis B virus: Galibert et al., 1979 Nature 281, 646-650.

Design of methods to screen agents

[0081] Methods to screen agents for their ability to inhibit translation initiation from a viral IRES sequence can be designed without detailed knowledge of the precise interaction between the viral and cellular materials involved, although such a knowledge can certainly be helpful. Many of the numerous methods described above to identify the presence of viral nucleic acid sequences which mediate preferential translation of viral RNAs, to identify cellular or other viral components involved, and to characterize the interactions between these components and the viral nucleic acid sequences, can be readily adapted to detect interference with the aforementioned interactions or with the effects of these interactions.

[0082] In accordance with the present invention, agents may be tested to determine their impact on the translation of a detectable reporter polypeptide from an RNA in which the coding sequence for the reporter is translationally linked to a viral nucleic acid sequence responsible for preferential translation of viral RNAs. Such assays were described in some detail above. Production of the detectable reporter polypeptide is examined under translation conditions in which such production is dependent upon the viral nucleic acid sequence. As a control, the chimeric RNA or a second RNA included in each test can include the coding sequence for a second detectable reporter polypeptide distinguishable from the first and translationally linked to RNA sequences responsible for ensuring normal translation of cellular mRNAs. Test agents is examined for their ability to interfere with the production of the reporter polypeptide linked to the viral nucleic acid sequence without affecting production of the reporter polypeptide linked to cellular translation sequences.

[0083] In some cases the translation conditions used for the test can be the translation conditions present in infected cells. In such cases the tests can be performed by introducing the chimeric RNA or a DNA sequence encoding it into cells which previously, concurrently or subsequently are also infected with the virus under study. The transfection-infection assay described in more detail below is an example of such a test. As an alternative to performing the test in intact cells, the translation conditions found in infected cells can be reproduced in *vitro* by preparing extracts from infected cells and adding these to or using them for *in vitro* translations of the chimeric RNAs.

[0084] In other cases it is not necessary to work with infected cells or extracts made from them, as for example in cases where the chimeric RNA can be constructed in such a way that production of the detectable reporter polypeptide is dependent on a viral nucleic acid sequence even in uninfected cells.

[0085] This is the case for a chimeric RNA in which production of the detectable reporter polypeptide requires initiation of translation at an internal site within the RNA. In other cases it may be possible to add an inhibitor to uninfected cells or extracts made from them which blocks a step or pathway normally blocked during viral infection. An example is the addition of cap analogs to inhibit cap-dependent initiation of translation.

[0086] Whichever approach is used, the tests can be performed in intact cells containing the chimeric RNAs, for example as the result of transcription of an appropriate DNA introduced into the cells, or by *in vitro* translation of these chimeric RNAs.

[0087] Detectable reporter polypeptides suitable for use in chimeric RNAs or control RNAs include, but are not limited to, easily assayed enzymes such as β-galactosidase, luciferase, β-glucuronidase, chloramphenicol acetyl transferase, and secreted embryonic alkaline phosphatase; proteins for which immunoassays are readily available such as hormones and cytokines; proteins which confer a selective growth advantage on cells such as adenosine deaminase, aminoglycoside phosphotransferase (the product of the neo gene), dihydrofolate reductase, hygromycin-B-phosphotransferase, thymidine kinase (when used with HAT medium), xanthine-guanine phosphoribosyltransferase (XGPRT), and proteins which provide a biosynthetic capability missing from an auxotroph; proteins which confer a growth disadvantage on cells, such as enzymes that convert non-toxic substrates to toxic products such as thymidine kinase (when used with medium containing bromodeoxyuridine) and orotidine-5'-phosphate decarboxylase (when used with 5-fluoroorotic acid); and proteins which are toxic such as ricin, cholera toxin or diphtheria toxin.

[0088] Transfection-infection assays can also be used to identify agents which interfere in the interactions between cellular or viral proteins or components and viral nucleic acid sequences responsible for preferential translation of viral RNAs. As described above, such assays involve the introduction into a cell by transfection of a gene or complementary DNA (cDNA) which encodes a detectable reporter polypeptide translationally linked to either a viral or a cellular translation sequence, and infection of this cell with the virus under study. Polypeptides linked to viral translation sequences are produced in greater quantities in infected cells than polypeptides linked to cellular translation sequences. Test agents can be screened for their ability to reduce or abolish this disparity without affecting the production of the reporter polypeptide linked to the cellular translation sequences.

[0089] It will be evident to one skilled in the art that transfection-infection assays can be replaced by similar assays in which stable cell lines are used which express appropriate reporter gene constructs. Such cell lines can be developed using selectable marker genes such as neo. With such a cell line the transfection step is eliminated, and assays would simply involve infection of the stable cell line with the virus.

[0090] In some cases the translation advantage conferred by a viral nucleic acid sequence may be so significant that it is observed even without viral infection, when that sequence is introduced artificially into a cell without other viral sequences. This is evidenced by superior translation in uninfected cells of a reporter polypeptide linked to the viral nucleic acid sequence as compared to the translation of the same polypeptide linked to a cellular translation sequence. In such cases, test agents may be screened in uninfected cells by determining their ability to reduce the enhanced translation of the reporter polypeptide linked to the viral sequence.

[0091] The above descriptions are provided by way of example and in no way limit the scope of the invention. It should be apparent that one skilled in the art is able to choose from a wide variety of methods to identify viral nucleic acid sequences responsible for preferential translation of viral RNAs, to identify other cellular and viral components involved, to characterize the interactions between the various partners which enable preferential translation of viral RNAs, and to develop tests which can be used to screen agents for their ability to disrupt or abolish such interactions.

[0092] The following are examples of methods used to screen for agents that block activity of translational control elements.

Screening IRES Elements

[0093] Developing assays to screen for agents that block IRES element activity preferably requires constructing a dicistronic mRNA characterized by the presence of two different reporter genes, wherein the translation of one gene is under IRES element control and translation of the other gene is under the control of the host-cell cap structure (m^7GpppG) and cellular 5'-UTR sequence. Such a construct makes it possible to identify agents, using either cell-free or cell-based assays, that block IRES element activity without adversely affecting the process that cells use to initiate translation of their own mRNA. Thus, the preferred embodiment of this invention enables the user to identify agents that have the desired mechanism of action while simultaneously eliminating nonspecific and possibly toxic agents.

[0094] The reporter genes can be any genes that encode products that can be conveniently and reliably detected. Commonly used detection methods include, but are not limited to, incorporation of radioisotopes, chemiluminescence, bioluminescence, colorimetric techniques and immunological procedures. Examples of appropriate reporter genes include luciferase, chloramphenicol acetyl transferase, secreted embryonic alkaline phosphatase, β-galactosidase, and dihyrodofolate reductase. This list is merely illustrative and in no way limits the scope of the invention since other suitable reporter genes will be known by those ordinarily skilled in the art. The method(s) for detecting the reporter gene products in the assay are preferably applied directly to the reactions or cells used to screen potential drug activity but, in a lesser embodiment, can also be used in conjunction with techniques for first fractionating the reaction mixtures. Said techniques, used either singly or in combination, may include chromatography, electrophoresis, filtration, ultrafiltration, centrifugation, precipitation, extraction, complex formation or digestion.

[0095] The dicistronic reporter gene construct can be used for either *in vitro* or *in vivo* agent screens. In the *in vitro* (cell-free) assay format, the dicistronic mRNA construct is encoded by a plasmid DNA molecule which directs transcription of the construct under the control of a strong promoter, exemplified by the bacteriophage T7 or SP6 promoters. When

purified and transcribed *in vitro* with the homologous RNA polymerase (e.g., T7 or SP6) in the presence of pre-formed cap structures, the plasmid directs the synthesis of large amounts of "capped" dicistronic reporter construct that can be purified using commonly practiced techniques. This dicistronic mRNA is then used as a template in a eukaryotic *in vitro* translation system either purchased from a commercial supplier or prepared according to procedures available in the scientific literature.

[0096] Agents may also be tested in whole cells that contain the above dicistronic reporter construct. Said construct is modified for use in cultured eukaryotic cells by: 1) placing the transcription of the construct under the control of a strong eukaryotic viral promoter, such as SV40, CMV or other promoters commonly used by those skilled in the art; 2) including splice signals such as SV40 splice signals to ensure correct processing and transport of RNAs made in the nucleus; and 3) including a polyadenylation signal such as the SV40 signal at the 3' end of the construct so that the reporter mRNA will be synthesized as a 3' polyadenylated molecule.

[0097] A plasmid encoding the dicistronic construct can be used to establish a transient expression assay for screening agents that block IRES activity or, in the preferred embodiment, to establish a stable cell line for screening agents. The latter may be accomplished by incorporating into the plasmid harboring the dicistronic reporter gene construct any of several commonly used selectable markers, such as neo, in order to select and maintain those cells containing the assay plasmid. Alternatively, a stable cell line can be generated by co-transfecting the desired host cells with two plasmids, one containing the selectable marker and the other containing the dicistronic reporter gene construct. Selecting for cells in a co-transfection procedure that have acquired one plasmid with a selectable marker is a commonly used way known to those skilled in the art to purify cells which have taken up a second plasmid which lacks the benefit of a selectable marker.

Libraries for screening

[0098] The assays encompassed by this invention can be used to screen agent libraries to discover novel antiviral drugs. Such libraries may comprise either collections of pure agents or collections of agent mixtures. Examples of pure agents include, but are not necessarily limited to, proteins, polypeptides, peptides, nucleic acids, oligonucleotides, carbohydrates, lipids, synthetic or semi-synthetic chemicals, and purified natural products. Examples of agent mixtures include, but are not limited to, extracts of prokaryotic or eukaryotic cells and tissues, as well as fermentation broths and cell or tissue culture supernates. In the case of agent mixtures, the assays are not only used to identify those crude mixtures that possess the desired antiviral activity, but also the assays provide the means to purify the antiviral principle from the mixture for characterization and development as a therapeutic drug. In particular, the mixture so identified can be sequentially fractionated by methods commonly known to those skilled in the art which may include, but are not limited to, precipitation, centrifugation, filtration, ultrafiltration, selective digestion, extraction, chromatography, electrophoresis or complex formation Each resulting subfraction can be assayed for antiviral activity using the original assay until a pure, biologically active agent is obtained.

[0099] In preferred embodiments, the assays designed for detecting antiviral activity are used for automated, high-throughput drug discovery screens in conjunction with the above mentioned libraries. The assays are performed in any format that allows rapid preparation and processing of multiple reactions such as in, for example, multi-well plates of the 96-well variety. Stock solutions of the test agents as well as assay components are prepared manually and all subsequent pipetting, diluting, mixing, washing, incubating, sample readout and data collecting is done using commercially available robotic pipetting equipment, automated work stations, and analytical instruments for detecting the signal generated by the assay. Examples of such detectors include, but are not limited to, spectrophotomers, colorimeters, luminometers, fluorometers, and devices that measure the decay of radioisotopes.

[0100] In another embodiment, the assays may be used to screen vast libraries of random peptides or oligonucleotides produced by any of the techniques already in the public domain or otherwise known to those skilled in the art. Because of their large size, these libraries are likely sources of lead agents since they can contain from 10^7 - 10^{10} chemical entities. Screening libraries of this size requires allowing test agents to bind to a molecular target *in vitro,* trapping the resulting complex in order to identify the specific lead agents that have been bound, and then producing the lead) agents in greater quantities for further development.

[0101] In the present invention, the molecular targets of choice comprise those segments of viral RNA that insure preferential translation of viral mRNA in virus-infected cells, as well as any viral or cellular protein(s) required by the viral RNA segment for this function. Either the assay target or the library agents are immobilized on a solid support so that the complexes formed between the molecular target and putative lead agents can be trapped and conveniently separated from unbound molecules. Amplification of the lead agents can be done chemically (peptide or oligonucleotide synthesis, respectively, once the sequence of the test agent has been deduced), enzymatically (PCR amplification reactions in the case of oligonucleotides) or biologically (propagation in *E. coli* of bacteriophage display vectors in the case of peptides). The lead peptide or oligonucleotide agents may be ultimately developed as drugs in and of themselves, or used for structural modeling studies to develop small molecule mimics which become the final drug.

[0102] The following broadly summarizes the main screening methods useful in this portion of the invention:

Cellular Assays

[0103] Assays that rely on whole cells can be used as primary screens or to screen compounds that pass the in *vitro* binding assays and cell-free translation assays. The cells to be used are first modified either stably or transiently (e.g. transfected) with selected reporter gene constructs. Either the monocistronic or dicistronic construct described in the preceding section is modified for use in cultured eukaryotic cells by: 1) placing the transcription of the construct under the control of a strong eukaryotic viral promoter, such as SV40, CMV or other promoters commonly used by those skilled in the art, 2) including splice signals such as SV40 splice signals to ensure correct processing and transport of RNAs made in the nucleus, and 3) including a polyadenylation signal such as the SV40 signal at the 3' end of the construct so that the reporter mRNA will be synthesized as a 3' polyadenylated molecule.

[0104] A plasmid encoding the construct can be used to establish a transient expression assay for screening compounds that block IRES activity or, in the preferred embodiment, to establish a stable cell line for screening compounds. The latter may be accomplished by incorporating into the plasmid harboring the desired reporter gene construct any of several commonly used selectable markers, such as neo, in order to select and maintain those cells containing the assay plasmid. Alternatively, a stable cell line could be generated by co-transfecting the desired host cells with two plasmids, one containing the selectable marker and the other containing the dicistronic reporter gene construct. Selecting for cells in a co-transfection procedure that have acquired one plasmid with a selectable marker is a commonly used way known to those skilled in the art to purify cells which have taken up a second plasmid which lacks the benefit of a selectable marker.

[0105] Also for the stable cell line assay, a reporter gene could be chosen and used, either for the monocistronic or dicistronic construct, that confers a growth advantage to cells exposed to a test compound chat inhibits IRES element activity. More specifically, the reporter gene placed under IRES element control could be a gene that encodes a product that inactivates, for example, a drug-resistance pathway in the cell or a pathway that confers resistance to any number of otherwise lethal environmental stresses (e.g. temperature, alcohol, heavy metals etc.). Cells containing this reporter gene construct grow poorly or not at all in the presence of the drug or stress, but if the same cells are treated with a test compound that inactivates the IRES element activity responsible for expression of the reporter gene, this gene product will not be made. Consequently, the pathway under its control will become active and enable the cells to grow in the presence of the environmental or drug insult.

[0106] The following examples of cell based assays illustrate, but in no way are intended to limit the present invention. The in vitro assays described below are provided by way of comparison.

Example 1 : Making/Isolating IRES Element RNA Constructs

A. *In Vitro* Transcription Reactions

[0107] Oligoribonucleotides are prepared by *in vitro* transcription from PCR templates amplified using a 5' primer containing a T7 promoter by procedures previously described (Milligan et al., 1987, Nucleic Acids Res. 15, 8783-8798.). RNAs are labeled by the addition of [a-^{32}P]-UTP (5 μCi) into the transcription reaction. Transcription reactions are purified using Stratagene NucTrap push columns and eluted with 5 mM Hepes pH 7.6, 25 mM KCl, 5 mM $MgCl_2$ and stored at -20°C.

B. PCR Reaction

[0108] Amplify selected IRES element from available plasmids using polymerase chain reaction (PCR) and primers designed to place T7 promoter on 5' end of PCR fragment. Reaction mixture contains the following: 1 μM primer #1, 1 μM primer #2, 40 μM dATP, 40 μM dGTP, 40 μM dCTP, 40 μM dTTP, 4 pg/μl template DNA, Taq DNA polymerase, 10. mM Tris-HCl pH 8.3 25°C, 40 mM KCl, 1.5 mM $MgCl_2$, and 0.01% (w/v) gelatin.

[0109] The reaction mixture (100 μl total volume) is overlaid with 100 μl mineral oil. Dip tube in mineral oil and place in heat block, forcing out air bubbles. Parameters: 94°C 2 minutes, 42°C 1 minute, 72°C 1 minute, 2 sec autoextension. Remove as much oil top layer as possible. Add 100 μl TE and extract with CHCl3, then phenol/CHCl3, and finally with CHCl3. Add 30 μl 3 M NaOAc. Add 600 μl ice-cold EtOH and let stand at -20°C for several hours. Spin 30 minutes at 14K rpm in microfuge, then resuspend in 5 μl H_2O.

C. Preparation of Internally Labeled IRES RNA for Filter Binding and UV Cross-Linking Assays

[0110] Reaction mixture contains the following components: 5 μl PCR fragment (5 μl), 0.1% DEPC H_2O (10 μl), 10

mM ATP (5 μl), 10 mM GTP (5 μl), 1 mM UTP (2.5 μl), 10 mM CTP (5 μl), [a-^{32}P]-NTP (100 μCi), RNasin (1 μl), and 5X buffer (10 μl; 200 mM Tris pH 8.0 37°C, 50 mM $MgCl_2$, 25 mM DTT, 1 mM spermidine, 40% PEG, 0.5% Triton X-100). The mixture is incubated at 37°C for 5 minutes, prior to addition of 4 μl T7 polymerase (1 mg/ml). The reaction mixture is then incubated at 37°C for 60 minutes. 2 μl RNase-free DNase is then added, and incubation continued at 37°C for 1 minute. The reaction is then terminated by the addition of 2 μl 500 mM EDTA and extracted with phenol/$CHCl_3$. Load transcription reaction on column (Stratagene NucTrap push column with 70 μl elution buffer (5 mM Hepes pH 7.6, 25 mM KCl, 5 mM $MgCl_2$). Elute RNA from push column with 70 μl elution buffer. Determine cpm/μl with scintillation counter, and store at -20°C. Check integrity of RNA on 6% acrylamide TBE 7M urea gel.

D. Preparation of End Labeled IRES RNA for Footprint Assay

[0111] A 500 μl T7 transcription reaction contains: PCR product (50 μl), 0.1% DEPC H_2O (320 μl), 100 mM ATP (5 μl), 100 mM GTP (5 μl), 100 mM UTP (5 μl), 100 mM CTP (5 μl), RNasin (5 μl), 5X buffer (100 μl: 200 mM Tris pH 8.0 37°C, 50mM $MgCl_2$, 25mM DTT, 5mM spermidine, 40% PEG, 0.05% triton X-100), 5' 37°C, T7 polymerase (1 mg/ml) and 5 μl 60' 37°C. 5 μl RNase-free DNase, 37°C 1 minute. Add 10 μl 500mM EDTA·phenol/$CHCl_3$ extract. Wash Stratagene NucTrap and push column with 70 μl elution buffer (5 mM Hepes pH 7.6, 25 mM KCl, 5 mM $MgCl_2$). Load transcription reaction on column. Elute RNA from Stratagene NucTrap, push column with 70 μl elution buffer. Add H2O to 180 μl and 20 μl 3M NaOAc pH 5.2. Add 600 μl ice-cold EtOH, then store at -20°C overnight. Spin down 14K rpm in microfuge at 4°C; read A260, then determine concentration. Store at -20°C.

[0112] To 5'-end-label RNA: dephosphorylate cold RNA with calf intestine alkaline phosphatase (0.1 unit/pmol end) in 50 mM NaCl, 10 mM Tris-HCl pH 7.9 (25°C), 10 mM $MgCl_2$, and 1 mM DTT. Incubate at 37°C for 60 minutes. Extract with phenol/$CHCl_3$, then $CHCl_3$ and EtOH precipitate. Phosphorylate RNA with T4 polynucleotide kinase and ^{32}P-ATP in 70 mM Tris-HCl pH 7.6 (25°C), 10 mM MgCl2, and 5 mM DTT, 37°C for 30 minutes. Extract with phenol/$CHCl_3$ then $CHCl_3$ EtOH precipitate, and resuspend in TE. Determine cpm/μl.

[0113] To 3'-end-label RNA: phosphorylate Cp with T4 polynucleotide kinase and ^{32}P-ATP in 70 mM Tris-HCl pH 7.6 (25°C), 10 mM $MgCl_2$, and 5 mM DTT, 37°C for 30 minutes. Ligate ^{32}P-pCp with cold RNA using T4 RNA ligase in 50 mM Tris-HCl pH 7.8 (25°C), 10 mM $MgCl_2$, 10 mM mercaptoethanol, and 1 mM ATP, 37°C 60 minutes. Extract with phenol/$CHCl_3$ then $CHCl_3$. EtOH precipitate, and resuspend in TE. Determine cpm/μl.

E. Construction of pBL and pBCRL Plasmids

[0114] Transcription template pBL was constructed by ligating PCR amplication products of β-globin and luciferase sequences into plasmid vector pUC19. β-globin PCR primers (SEQ. ID NO. 18, SEQ. ID NO. 19) were designed to amplify the 5' non-translated region ("NTR" also referred to as untranslated region, "UTR") of β-globin and introduce a 5' EcoR I restriction site, a 5' T7 promoter, and a 3' Kpn I restriction site. The EcoR I and Kpn I restriction sites were used for ligation into pUC19 to generate the intermediate plasmid pB. Luciferase PCR primers (SEQ. ID NO. 20, SEQ. ID NO. 21) were designed to amplify the luciferase coding sequence and introduce a 5' Pst I restriction site and a 3' Hind III restriction site, for ligation into pB to generate pBL. CAT PCR primers (SEQ. ID NO. 22, SEQ. ID NO. 23) were designed to amplify the CAT coding sequence and introduce a 5' Kpn I restriction site and a 3' Bam HI restriction site, for ligation into pBL to generate pBCL. Rhinovirus 14 5' NTR PCR primers (SEQ. ID NO. 24, SEQ. ID NO. 25) were designed to amplify the rhinovirus 5' NTR and introduce a 5' Bam HI restrictions site and a 3' Pst I restriction site which were used to ligate the amplification product into pBCL. Rhinovirus and luciferase start codons are aligned by transforming the resultant plasmid containing β-globin 5' NTR, CAT, rhinovirus IRES and luciferase sequences into E. coli DMI cells. Unmethylated plasmid DNA is isolated and digested with Bcl I, the digested plasmid was religated and transformed into E. coli DH5 cells to produce pBCRL.

F. Ligation Reaction, Plasmid Screening, and Purification

[0115] DNA fragments were purified on low melting point agarose gels (Maniatis et al., 1989, In: Molecular Cloning: A Laboratory Manual, cold Spring Harbor, New York) and ligated with T4 DNA ligase in a 10 μl reaction in 10 mM Tris-HCl pH 7.9 (25°C), 10 mM $MgCl_2$ 50 mM Nacl, 1 mM DTT, and incubated overnight at 16°C. Ligated plasmids are transformed into *E.coli* DH5 or DMI bacterial host cells using rubidium chloride treatment. Transformants harboring plasmid DNA were screened by ampicillin resistance and restriction analysis of minilysate plasmid DNA (Maniatis et al., 1989, In: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York). Plasmids were sequenced in the region of interest with T7 DNA polymerase using ^{35}S-labeled dATP.

G. Purification of DNA from LMP Agarose

[0116] Load cut DNA onto 1% LMP agarose gel in TAE with 0.5 ug/ml EtBr. Run gel slowly (25 mA for several hours) for maximum resolution and to avoid melting. Take picture and locate bands to cut out. Quickly cut out band of right size and put in Eppendorf tube. Add 10 µl 1 M Tris-HCl pH 8.0, 10 µl 8 M LiCl, bring volume to approximately 200 µl with H_2O. Add 200 µl phenol (not phenol/$CHCl_3$). Melt agarose 70°C for 5 minutes. Spin 14 K rpm 5 minutes (white interphase appears). Remove aqueous phase and phenol extract again at 70°C (clear interphase). Extract with 200 µl $CHCl_3$ twice at 25°C. Add 400 µl EtOH and keep -20°C 1 hour. Spin down, dry pellet, dissolve in 10 µl TE. 10 X TAE buffer: 24.2 g Trizma Base, 5.7 ml glacial acetic acid, 12.5 ml 0.4 M EDTA, bring up to 500 ml with H_2O.

H. Ligation

[0117] pBLuc Construction: Ligate 0.1 ug PUC18 (digested with KpnI and SalI) with PCR1 (digested with KpnI and ApaI) and PCR2 (digested with ApaI and SalI).

[0118] pBCATIRESLuc Construction: Ligate 0.1 ug pBLuc (digested with XhoI and BclI) with PCR3 (digested with XhoI and NheI) and PCR4 (digested with NheII and BclI) .

I. Transformation

[0119] Preparation of Competent Cells: Grow 5 ml of DH5 cells overnight 37°C. 2 mls overnight into 100 mls LB in 500 ml flask. Grow to OD = 0.48 A600 (around 2 hours). Split into two 50 ml fractions and spin in SS34 rotor 5 minutes at 4800 rpm, 4°C. Decant supernatants and resuspend by vortexing each fraction in 16 mls Rb1. Combine tubes, then spin in SS34 rotor 10 minutes at 4800 rpm, 4°C. Decant supernatant. Gently resuspend cell pellet in 3.2 mls of Rb2 15 minutes 4°C. Quick freeze 200 µl aliquots and store -80°C.

Rb1	MW	for 200 mls
30mM KOAc	98.14	589mg
100mM $RbCl_2$	120.9	2.42g
10mM $CaCl_2$-H_2O	147.02	294mg
50mM $MnCl_2$-$4H_2O$	197.9	1.98g .
15% glycerol		30mls

Adjust pH to 5.8 with 0.2 M acetic acid (5.75 mls in 500 mls). Filter sterilize.

Rb2	MW	for 200 mls
10mM MOPS	209.3	209 mg
10mM $RbCl_2$	120.9	120mg
75mM $CaCl_2$-H_2O	147.02	1.1g
15% glycerol		30mls

Adjust pH to 6.5 with 1M KOH. Filter sterilize.

[0120] Transformation: 100 µl competent cells plus DNA. 30 minute 4°C. Heat shock 2 minutes 42°C. Place back on ice, and add 1 ml LB broth (best to transfer to culture tube containing 2ml LB broth). 37°C 1 hr with shaking plate 100 µl on selective plate. Spin down remaining cells, decant, resuspend, and plate on selective plate.

J. DNA Sequencing with USB Sequenase Kit

[0121] Extract (mini-prep) DNA from 1.5 ml overnight (or 1 ug purified DNA). Resuspend in 25 µl TE with RNase A. Put 8 µl of DNA into new tube, and add 2 µl 2M NaoH; 2mm EDTA 5 minutes 25°C. Add 7 µl primer DNA (2pmol/µl). Add 3 µl 2M NaOAc pH 4.6. Mix gently, then add 75 µl EtOH. 45 minutes -80°C (overnight OK). Spin 15 minutes in microfuge. Dry pellet. Dissolve, pellet in 8 µl dH_2O, add 9 µl sequence cocktail and incubate 2 minutes 25°C. Dispense 3.5 µl of mixture into four tubes, each containing 2.5 µl ddNTP termination mix. 15 minutes 37°C. Add 4 µl stop solution. Boil 3 minutes. Load 3 µl on 6% acrylamide, 7 M urea gel.

Cocktail	2 rxns-far	3 rxns-far	5 rxns-far	5 rxns-close
seq buffer	4	6	10	10
0.1M OTT	2	3	5	5
dGTPlabel mix	0.8	1.2	2	10 (1/20)
355-dATP	2	3	5	5.0
H2O	13	14.4	26	12.0
Sequenase®	0.5	0.8	1.1	1.1
Mn buffer				5.0

K. Preparation of Capped RNA for Translation Reactions

[0122] T7 polymerase transcription from plasmid DNA was as follows.

[0123] A 200 μl reaction contains: 5 ug plasmid, 1 mM each NTP, 5 ug cut plasmid DNA (20 μl), 0.1% DEPC H_2O (128 μl), 100 mM ATP (2 μl), 10mM GTP (2 μl), 100mM UTP (2 μl), 10 mM m^7GpppG (20 μl), RNasin (1 μl), 5X plasmid buffer (40 μl), incubate 5' 37°C. Add 4 μl polymerase (2-4 μl), incubate 60' 37°C. Add 10 μl RNase-free DNase, incubate 37°C for 1 minute. Add 5 μl 500mM EDTA. Phenol/$CHCl_3$ extract. $CHCl_3$ extract. Add 70 μl 0.1% DEPC H_2O. Add 30 μl 3M NaOAc pH 5.2. 900 μl EtOH. -20°C overnight or -80°C 30 minutes, resuspend in 25 μl TE. Read A_{260}, Transcription Buffer: 200 mM Tris pH 8.0 at 37°C, 50 mM $MgCl_2$, 25 mM DTT, 5 mM spermidine, 250 ug/ml BSA, 0.1% DEPC H_2O (650 μl), 1M Tris (200 μl), (pH 8.0 @ 37°C, pH 8.4 @ 25°C), 1M DTT (25μl), 100 mM spermidine (50 μl), 10 ug/μl BSA (25 μl), 1M $MgCl_2$ (50 μl), store - 20°C (1000 μl).

L. Construction of Mono- and Dicistronic Plasmids for Transfection Assays

[0124] A dicistronic plasmid (pCMV-Luc-IRES-SEAP) is used to transfect cells and assay for translation in vivo in the presence and absense of test compounds. pCMV-Luc-IRES-SEAP contains, in order, the SV40 replication origin, cytomegalovirus (CMV) promoter, luciferase reporter gene, selected IRES element, secreted alkaline phosphatase (SEAP) reporter gene, SV40 splice sites, and SV40 polyA signal. Two pUC118-based constructs (pB-SEAP and pB-Luc-IRES-SEAP) are used to construct pCMV-Luc-IRES-SEAP. pB-SEAP contains, in order, a T7 polymerase promoter, β-globin 5' nontranslated region, and SEAP reporter gene. pB-Luc-IRES-SEAP is constructed from pB-SEAP and contains, in order, a T7 polymerase promoter, β-globin 5' nontranslated region, luciferase reporter gene, selected IRES element, and SEAP reporter gene. Construction of pB-SEAP and pB-Luc-IRES-SEAP is performed by PCR amplification of β-globin 5' NTR, luciferase coding sequence, IRES element, and SEAP coding sequence from available plasmids using primers containing unique 5' restriction sites. PCR products containing the β-globin 5'NTR and SEAP coding region are restriction digested and inserted into PUC118 to produce the monocistroic construct pB-SEAP. The dicistronic plasmid pB-Luc-IRES-SEAP is created by ligating the restriction digested monocistronic plasmid and restriction digested PCR products containing the selected IRES element and luciferase coding region. The dicistronic plasmid used to transfect cells (pCMV-Luc-IRES-SEAP) is constructed by ligating a blunt-ended Kpn I and Apa I fragment containing the LUC-IRES-SEAP coding region of pB-LUC-IRES-SEAP and Eco RV-digested plasmid vector pcDNAI-neo (InVitrogen) containing cytomegalovirus (CMV) promoter, containing SV40 replication origin, splice sites, and polyA signal.

M. PCR

[0125] Amplify T7 promoter, β-globin 5' NTR, luciferase reporter gene, IRES element, and SEAP reporter gene using polymerase chain reaction (PCR) described above and primers shown below.

PCR Product	5' Primer	3' Primer	Sequence
1	GW2	GW3	T7-β-globin 5'NTR
5	GW10	GW11	SEAP
4	GW8	GW9	IRES element
6	GW12	GW13	luciferase

Example 2 : Filter Binding Assays for IRES-Binding Proteins

[0126] Polypyrimidine tract binding protein (pPTB, p57; Jang and Wimmer, 1990, Genes Dev. 4, 1560-1572; Pestova et al., 1991, J. Virol. 65, 6194-6204.; Luz and Beck, 1991, J. Virol. 65, 6486-6494.; Borovjagin et al., 1990, FEBS Lett. 2, 237-240.), La (p52), eIF2/2B (Scheper et al., 1991, Biochem. Biophys. Acta 1089, 220-226.), and p70 and p100 have been identified as IRES binding proteins. Filter binding assays for pPTB have been established and are described below. Filter binding conditions for the other purified proteins must be determined. IRES elements targeted include those from rhinovirus, coxsackievirus, poliovirus, echovirus, hepatitis A virus, hepatitis B virus, hepatitis C virus, mengo virus, encephalomycarditis virus, foot and mouth disease virus, theiler's murine encephalomyelitis virus, infectious bronchitis virus, vesicular stomatitis virus, and sendai virus.

[0127] Polypyrimidine Tract Binding Protein (pPTB) is purified from *E. coli* as a recombinant product which contains 12 amino acids from the expression vector fused to the pPTB amino terminus. Protein-excess filter binding assays are performed as follows: typical 25 μl reactions contain ^{32}P-internally labeled IRES element, pPTB, and MMK buffer (50 mM MES, pH 5.5, 10 mM KCl, 5 mM MgOAc) and are incubated at 25°C for 10-30 min before filtration in the presence or absense of test compound. Reactions are filtered through Schleicher and Schuell nitrocellulose filters (0.45 μm pore size) presoaked in MMK buffer. The filters are then washed with 200 μl of MMK buffer, dried in scintillation vials for 20 min at 190°C, and counted in Econolume. All RNAs are heated to 95°C for 3 min and quick cooled on ice just before use. Backgrounds obtained in the absence of protein are less than 5% of the input radioactivity and subtracted in all cases. Filtration assays contain ^{32}P-labeled RNA (~10 pM) and pPTB concentrations from 5 nM to 100 nM. Retention efficiencies of the RNA range from 40% to 60%. Equilibrium binding constants vary less than a factor of two for independent replicates.

Establishment of Filter Binding Assays for Other IRES Binding Proteins

[0128] (Purified La, eIF2/2B, p70, and p97 are incubated with 32P-internally labeled IRES elements under various solution conditions with pH ranges from 4-9, temperature ranges from 4-50°C, monovalent salt (Li^+, Na^+, K^+, R^b+) concentrations from 0-500mM, divalent salt Be^{++}, Mg^{++}, Ca^{++}, Ba^{++}) concentrations from 0-50mM, with counter anion F-, Cl-, Br-, I-, and OAc-.

Example 21: Chemical Methods for Detecting IRES-Binding Proteins

Footprint Assays

[0129] 5' or 3' end labeled RNA is incubated with purified pPTB, La, eIF2a, p70 or p97 protein under conditions which allow binding and nuclease activity. Ribonuclease T1 or S1 is added at a determined concentration, temperature, and time to give 1 hit/molecule RNA. Reactions are quenched by adding 7 M urea and quick freezing in dry ice-EtOH bath. Digested RNA fragments are separated on a 6% acrylamide, 7 M urea slab gel. Digestion in absence of protein produces a ladder of RNA digestion products; protection of RNA from nuclease by protein is observed as missing bands in ladder. Test compounds which interfere with interaction will restore ladder of RNA digestion products.

Cross-Linking Assays

[0130] Ultra-violet light cross-linking assays were performed as described previously (Jang and Wimmer, 1990, Genes Dev. 4, 1560-1572). ^{32}P-labeled RNAs were incubated with 50 μg of HeLa extract in 30 μl of cross-link buffer (5 mM Hepes pH 7.6, 25 mM KCl, 5 mM $MgCl_2$, 3.8 % glycerol) containing 1 μg rRNA at 30°C for 20 minutes. Reactions were cross-linked in a Stratagene cross linker for 40 minutes. RNAs were digested by incubation with 20 μg RNaseA and 200 units of RNase T1. Cross-linked proteins were separated on 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gels using the buffer system of Laemmli (1970, Nature 227, 680-685.), as modified by Nicklin et al., (1987, Proc. Natl. Acad. Sci. USA 84, 4002-4006.). Gels were electrophoresed at 5-10 volts/cm at constant current (70 mA), dried, and autoradiographed. The intensity of the cross-linking signal was quantitated by scanning densitometry.

Example 3 : In vitro Translation Screening Assays

[0131] Test compounds are screened for their ability to inhibit viral IRES-directed protein translation in a cell-free system containing an IRES element-protein coding region-containing construct, the selected cellular binding protein required for viral translation, and cellular translation components (ribosomes, etc.).

A. *In Vitro* Translation Assay

[0132] Two pUC118-based constructs (pBL and pBCRL, described above) are used to assay for translation in the presence and absense of test compounds. pBL contains, in order a T7 polymerase promoter, β-globin 5' nontranslated region, and luciferase reporter gene. pBCRL contains, in order, a T7 polymerase promoter, β-globin 5' nontranslated region, CAT reporter gene, IRES element, and luciferase reporter gene. Test compounds are screened for their ability to inhibit luciferase synthesis driven by an IRES element using construct pBCRL, but not CAT synthesis driven by a β-globin 5'NTR using construct pBCRL and not luciferase synthesis driven by β-globin 5'NTR using construct pBL.

[0133] IRES elements targeted include those from rhinovirus, coxsackievirus, poliovirus, echovirus, hepatitis A virus, hepatitis B virus, hepatitis C virus, mengo virus, encephalomycarditis virus, foot and mouth disease virus, theiler's murine encephalomyelitis virus, infectious bronchitis virus, vesicular stomatitis virus, and sendai virus.

B. Preparation of S10 of Hela S3 for Translation : Materials/Preparations

[0134] Rinse Type B homogenizer with EtOH and DEPC H_2O in hood. Hypotonic Lysis Buffer: 0.119 g Hepes (500 μl 1M), 0.049 g KOAc (250 μl 2M), 0.016 g MgOAc (74 μl 1M), DEPC H_2O to 50 mls, adjust pH to 7.4 with 1 M KOH. Add 25 μl 1 M DTT in 10 ml Hepes buffer, prepare fresh. Dialysis Buffer: 2.383 g Hepes, 8.833 g KOAc, 1.5 ml 1 M MgOAc, H_2O to 1 L (non-DEPC H_2O will suffice). Adjust pH to 7.4 with 1 M KOH, add 25 ml 1 M DTT in 10 ml Hepes buffer. Autoclave or filter sterilize and store 4°C. Dialysis tubing 12000-14000 cutoff. 2X Load Dye: 125 μl 1 M Tris-HCl pH 6.8, 400 μl 10% SDS, 100 μl mercaptoethanol, 375 μl 50% glycerol. Add trace bromophenolblue.

[0135] Obtain 2L HeLa S3 cells that are in log-phase (5X105 cells/ml). Wash cells 3 times with ice-cold PBS: (20 ml PBS (10ml PBS/L cells) for 1st wash, 15 ml PBS/L cells for 2nd wash, and 10 ml PBS/L cells for 3rd wash. Spin 2K rpm 10 minutes. Use 30ml corex tube and HB4 rotor for third spin. Resuspend to 1.5X packed cell volume with hypotonic buffer and swell on ice 10'. Hypotonic buffer (RNase free): 10 mM K-HEPES pH 7.4 1M stock, 10 mM KOA 4 M stock, 1.5 mM MgOAc (1 M), stock 2.5 mM DTT (add just before use). Homogenize with 15-45 strokes of type B homogenizer. Check cell disruption either visually or by dye exclusion assay after 10, 15, 20, 25 etc. strokes. If cells disrupted will see debris. Spin 5 minutes 2 K rpm (remove nuclei). Take supernate and spin 20' at 10 K rpm. Use sterile corex tubes. Dialyze 2 hours against 1 L (100 volumes) dialysis buffer (10 mM K-Hepes, pH 7.5, 90 mM KOAc, 1.5 mM MgOAc, 2.5 mM DTT) to clean and replace buffer. Add 2.5 ml 1 M DTT just before use. Freeze at -80°C overnight, thaw at 25°C approximately 30 minutes, immediately place on ice. Spin 10 K rpm for 10 minutes in microfuge. Add 200 μl 50% glycerol/ 800 μl lysate supernatant. Add 7.5 μl (2mg/ml) micrococcal nuclease and 7.5 μl 100mM CaCl, per 1 ml extract. Incubate 25°C 15 minutes. Add 15 μl 200mM EGTA/ml extract. Aliquot 150 μl/tube, store -80°C.

C. Translation Reaction

[0136] 10X Translation Mix: 1 mM ATP, 50 μM GTP, 10 mM creatine phosphate, 24 μg/ml CPK, 18 mM Hepes, 2 mM DTT, 24 μg/ml tRNA, 12 μM amino acid mix, 240 μM spermidine. Aliquot and store at -80°C. Mixture contains the following: 40 μl 100 mM ATP, 6 μl 40 mM GTP, 40 μl 1 M creatine phosphate (store -20°C), 10 μl 10 mg/ml creatine phospho kinase in Hepes (store -20°C), 76 μl K-Hepes pH 7.6, 8 μl 1 M DTT (thaw at 37°C), 10 μl 10 mg/ml calf liver tRNA (Boehringer), 50 μl amino acid mix-methionine, 10 μl 100 mM spermidine, and 250 μl H2O to 500 μl.

[0137] Master Mix (Prepare Fresh): Mixture contains: 150 μl micrococcal nuclease treated HeLa extract, 50 μl translation mix, 22 μl 2 M KOAc, 3 μl 50 mM MgOAc, 16 μl 20 mM MgCl2, 25 μl 35S-met (20 μCi/μl), sufficient for 28 translations, for fewer samples take less.

[0138] Translation: Mixture contains: 8.0 μl master mix, 4.5 μl 1 uM RNA in DEPC H_2O, +/- 10 μl test compound, incubate 30°C 3 hours. Add 40 μl 2X load dye, 28 μl H_2O, boil 5 minutes, load 20 μl on 12% gel, fix, enhance, expose to XRP film. Try 1M sodium salicylate 16g/100ml to enhance.

D. Luceriferase Assay

[0139] As described by DeWet et al., (1987, Mol. Cell Biol. 7, 725-737.). Prepare 1 mM stock solution of D-Luciferin by adding 2.8 mg luciferin (free acid - keep on ice and dark) to 9.8 ml H_2O, vortex to remove clumps, add 100 μl 1M Na_2HPO4 (gives yellow-green color, some precipitate maybe) add 100 μl 1M NaH_2PO4-H_2O (solution clears); aliquot and store at -20°C. Prepare stock of luciferase in H_2O at 1-10mg/ml, aliquot, store -20°C. Commercial luciferase dissolved at <lmg/ml in tricine buffer, DTT, $MgSO_4$, and 0.1% BSA, aliquot, store -20°C. Store transfected cells (not lysed) at -20°C. 100 μl lysate aliquot, store 4°C 2-4 weeks. *In vitro* translation, store -20°C. To perform assays, use 350 μl assay buffer at 25°C, add 10-50 μl cold cell supernatant from 100 μl lysate, or 1-10 μl from 20 μl *in vitro* translation reaction. Inject 100 μl luciferin solution. Assay Buffer (use fresh): 125 μl 100mM ATP, 75 μl 1M MgSO4, 4675 μl sonication buffer (100mM K_2HPO4 [dibasic] pH 7.8, 1mM DTT).

E. Cellular assay

[0140] A dicistronic construct directing synthesis of two different reporter proteins is transfected into cells; cells are exposed to test compounds, then are tested for their ability to produce each of the reporter proteins. Production of both reporter proteins is visualized or detected in the same cell preferably simultaneously or alternatively sequentially. The reporter proteins may be any of luciferase, β-galactosidase, secreted embryonic alkaline phosphatase, CAT, β-glucuronidase or other suitable protein as is known in the art.

[0141] Compounds that selectively inhibit viral translation inhibit production of reporter protein 2, but not reporter protein 1; compounds that are generally toxic to cells inhibit the synthesis of reporter protein 1 and possibly reporter protein 2.

Example 4 : Inhibiting Rhinovirus Translation with Antisense DNA Oligonucleotide Inhibitors.

[0142] The rhinovirus IRES-dependent translation system is an excellent target for antiviral compounds since it is essential for rhinovirus infection and very different than conventional human cellular translation systems. A screening assay for rhinovirus IRES-dependent translational inhibitors has been established by Applicant and the rhinovirus 14 IRES has been shown to be functional *in vitro.* Using this assay system, Applicant has identified antisense deoxyoligonucleotides that specifically inhibit rhinovirus IRES-dependent translation.

A. Rhinovirus translation

[0143] Translational initiation of rhinovirus mRNA has been shown to occur by a cap-independent non-scanning mechanism, in which the 40S ribosome locates the correct start codon by binding directly to a region of the viral 5' NTR, termed the internal ribosomal entry site (IRES) (Borman and Jackson, 188 Virology 685, 1992). Similar IRES-dependent translational initiation mechanisms have been proposed for other picornaviruses including poliovirus (Pelletier and Sonenberg, 334 Nature 320, 1988, and 63 J. Virol. 441, 1989), EMCV (Jang et al., 62 J. Virol. 2636, 1988, and 63 J. Virol. 1651, 1989; Molla et al., 356 Nature 255, 1992), FMDV (Kuhn et al., 64 J. Virol. 4625, 1990), HAV (Brown et al., 65 J. Virol. 5828, 1991), and an enveloped plus-strand RNA virus, hepatitis C virus (Tsukiyama-Kohara et al., 66 J. Virol. 1476, 1992).

[0144] Rhinovirus belongs to the picornavirus family. The secondary structures of several picornavirus IRES elements, as well as the hepatitis C virus IRES element, have been proposed (Pilipenko et al., 168 Virology 201, 1989a, and 17 Nucleic Acids Res. 5701, 1989b; Tsukiyama-Kohara et al., 66 J. Virol. 1476, 1992). On the basis of their nucleotide sequences and proposed secondary structures, IRES elements of picornaviruses can be divided into three groups; group I belonging to the genera Enterovirus and Rhinovirus, group II belonging to the genera Cardiovirus and Aphthovirus, and group III belonging to the genus Hepatovirus of the Picornaviridae family (Jackson et al., 15 Trends Biochem. Sci. 477, 1990). Remarkably, the IRES elements between the three groups share little sequence or structural homology, and none of the IRES elements from the three picornavirus groups resemble the IRES element of hepatitis C virus. The boundaries of the rhinovirus 2, poliovirus 2, and EMCV IRES elements have been determined by making 5' and 3' deletions of the IRES elements and assaying for cap-independent translation (Borman and Jackson, 188 Virology 685, 1992; Nicholson et al., 65 J. Virol. 5886, 1991; Jang and Wimmer, 4 Genes Dev. 1560, 1990). The boundaries determined indicate that all picornavirus IRES elements are approximately 400 nucleotides ("nts") long. Although the boundaries of the rhinovirus 14 IRES have not yet been determined, by extrapolating from the above results, it is likely that the 5' border is near nt 117 and the 3' border is near nt 577 (Figure 5).

[0145] Oligopyrimidine tracts have been found near the 3' border of all picornavirus IRES elements (Figure 5, nt 572-580). Closer inspection of the various oligopyrimidine tracts revealed the presence of a downstream AUG triplet (Figure 5, nt 591-593). This conserved element has been termed the "Y_nX_mAUG" motif, with Y_n corresponding to a pyrimidine tract of length n, wherein n may vary from 4 to 12 and most preferably from 5-9 nucleotides, and X_m corresponding to a random spacer sequence of length m, wherein m may vary from 5 to 30 and most preferably 10-20 nucleotides (Jang et al., 44 Enzyme 292, 1990). Site directed and genetic alterations of the "Y_nX_mAUG" motif suggest that the sequence of the pyrimidine tract and AUG sequence are important for IRES function, as well as proper spacing between the pyrimidine tract and the AUG (Pelletier et al., 62 J. Virol 4486, 1988; Pestova et al., 65 J. Virol. 6194, 1991; Pilipenko et al., 68 Cell 1, 1992). The "Y_nX_mAUG" motif has been proposed to unify cap-independent translation among picornaviruses and may be involved in 18S ribosomal RNA binding (Jang et al., 44 Enzyme 292, 1990; Pilipenko et al., 68 Cell 1, 1992). In rhinoviruses and enteroviruses there is also a conserved 21 base sequence found upstream of the "Y_nX_mAUG" motif. It will be evident to one skilled in the art that in the design of an antisense oligonucleotide effective in inhibiting translation the oligonucleotide will be complementary to sequences at least partly within the IRES, and such sequences will be attractive targets for antisense oligonucleotides. The importance of this sequence in IRES-dependent translation is unknown.

[0146] The start codon used by the rhinovirus IRES element is located approximately 31 nucleotides downstream of the "Y_nX_mAUG" motif. It has been proposed that for rhinoviruses the ribosome binds the IRES element and then scans to the authentic start codon of the polyprotein (Jackson et al., 15 Trends Biochem. Sci. 477, 1990; Jang et al., 44 Enzyme 292, 1990).

[0147] Several cellular proteins have been observed to bind IRES elements or fragments of IRES elements (Witherell et al., 32 Biochemistry 8268, 1993; Borman et al., 74 J. Gen. Virol. 1775, 1993; Meerovitch and Sonenberg, 4 Seminars Virol. 217, 1993; Witherell and Wimmer, J. Virol., in press 1994). For some of these proteins there is also evidence of a functional role in cap-independent translation (Jang and Wimmer, 4 Genes Dev. 1560, 1990; Borman et al., 74 J. Gen. Virol. 1775, 1993; Meerovitch et al., 67 J. Virol. 3798, 1993). Two cellular proteins have been found to act synergistically to stimulate cap-independent translation directed by the rhinovirus IRES element (Borman et al., 74 J. Gen. Virol. 1775, 1993).

B. *In vitro* translation assay

[0148] To assay translation that is dependent upon the rhinovirus IRES element *in vitro*, the dicistronic mRNA (bCRL) is prepared containing the β-globin 5' NTR driving translation of the CAT reporter gene and rhinovirus IRES driving translation of the luciferase reporter gene (Figure 6A). Translational initiation of the CAT reporter in the dicistronic mRNA will be cap-dependent, whereas translational initiation of the luciferase reporter is dependent on the rhinovirus IRES. A compound that inhibits luciferase expression, without concomitant inhibition of CAT expression, indicates a selective block of IRES-dependent translational initiation. A control monocistronic mRNA is prepared (bL) containing the β-globin 5' NTR driving translation of the luciferase reporter gene (Figure 6B). bL mRNA is used as a control to screen out compounds that inhibit luciferase activity by inhibiting translational elongation or termination of the luciferase reporter gene, shifting the ribosome out of frame, or directly inhibiting enzymatic activity of the luciferase gene product. bL and bCRL mRNAs are produced by *in vitro* transcription from plasmids pBL and pBCRL (not shown) using T7 RNA polymerase (Milligan et al., 15 Nucleic Acids Res. 8783, 1987).

[0149] There are several different ways to quantitate luciferase activity. Translation reactions can be performed in HeLa extract, or other cell lines, as described by Sonenberg and co-workers (Lee and Sonenberg, 79 Proc. Natl. Acad. Sci. USA 3447, 1982) . Translations are performed with or without micrococcal nuclease treatment of the extracts under optimal conditions for rhinovirus IRES-dependent translation. All components of the reaction, including antisense deoxyoligonucleotides, are added to the translation reaction prior to the mRNA. No artificial annealing conditions for binding the antisense deoxyoligonucleotides and mRNA (i.e., high DNA and RNA concentrations, high salt concentrations, or heating and cooling steps) are required. An enhanced luciferase assay kit (available from Analytical Luminescence Laboratory, Promega, or other companies) is used to quantitate luciferase activity. In this assay, the translation reaction is performed in a well of the microtiter plate at 30°C for 3 hrs. Buffer(s) from the enhanced luciferase assay kits are added, the sample mixed, and the light emitted from the reaction quantitated by a luminometer or scintillation counter. The luciferase signal from translation of mRNA is typically >10,000-fold above the background signal (-mRNA). As an alternative to a commercial luciferase assay kit, a non-enhanced assay described by DeWet et al (1987) could be used. Luciferase and CAT expression, from in *vitro* translation reactions with HeLa extract, can also be quantitated by a [^{33}S]-methionine incorporation assay. [^{35}S]-Methionine incorporation is measured by translating bCRL and bL mRNA in the presence of [^{33}S]-methionine, separating the proteins by SDS-PAGE, and visualizing the bands by autoradiography.

[0150] A transient transfection assay can also be employed using bCRL mRNA and bL mRNA or pCMV-LUC and pCMV-LUC-IRES-SEAP plasmid DNA. bCRL and bL mRNA or pCMV-LUC and pCMV-LUC-IRES-SEAP plasmid DNA is introduced into HeLa cells, or other cell lines such as 293 or Jurkat, using lipofectin (Gibco, Inc.), electroporation, or DEAE dextran methods. Luciferase activity from in *vivo* translation of bCRL and bL mRNA is measured by preparing cell extracts using either the triton X-100 or freeze/thaw method and quantitating light emission. Alternatively, luciferase assays may be performed by growing transiently transfected cells in a microtiter plate and using a 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane (DMNPE) caged luciferin substrate (Yang and Thomason, 15 BioTechniques 848, 1993). DMNPE caged luciferin is generated in a simple one-tube synthesis and requires no further purification. The caged luciferin readily crosses the cell membrane and is cleaved by endogenous esterases, trapping the luciferin substrate in the cell. Light output from the cells is proportional to luciferase expression and is quantitated with the luminometer.

[0151] The rhinovirus 14 IRES of bCRL was shown to be functional in the HeLa extract translation system using a ^{35}S-methionine incorporation assay. Translation of dicistronic bCRL mRNA was compared to translation of a dicistronic mRNA, bCXL, containing a reversed and complementary sequence to the rhinovirus IRES. The translation efficiency of luciferase from bCRL mRNA (driven by the rhinovirus IRES) is as great as the translation efficiency of CAT driven by the β-globin 5' NTR (Figure 7, compare luciferase translation and CAT translation in lanes 7 and 8). Translation of luciferase from dicistronic bCXL mRNA, containing a reversed and complementary IRES, is however barely detectable. As an internal control, translation of CAT (driven by the b-globin 5' NTR) from bCXL is equivalent to translation of CAT from bCRL. Like the rhinovirus IRES element, the reversed and complementary IRES is predicted to form a high degree

of secondary structure that would make scanning through this region unlikely (Jackson et al., 15 Trends Biochem. Sci. 477, 1990). Luciferase translation from bCRL is therefore dependent on the presence of the IRES in the correct orientation and cannot be due to RNA degradation or alternative translational initiation mechanisms such as termination-reinitiation, leaky scanning, or ribosome jumping. These results provide strong evidence that the rhinovirus IRES in bCRL is functional.

C. Antisense oligodeoxynucleotide results

[0152] Applicant has designed antisense deoxyoligonucleotides that target the 3' end of the rhinovirus IRES element and inhibit rhinovirus IRES-dependent translation. This region of the IRES was chosen since it contains both the "Y_nX_mAUG" motif and the conserved 21 base sequence described above and shown in Figure 1. Antisense deoxyoligonucleotide inhibition of the rhinovirus IRES element was assayed using the [^{35}S]-methionine incorporation assay (Figure 7) and luciferase activity assay (Figure 8). An example of an antisense oligonucleotide that targets this region is anti-IRES-oligo, which anneals to nts 518-551 of the rhinovirus 14 IRES. The sequence of anti-IRES-oligo (SEQ. ID NO. 26) is

5' AGTAGTCGGTCCCGTCCCGGAATTGCGCATTACG 3'

[0153] Translation of monocistronic bLuc mRNA (Figure 6A) and dicistronic bCRL mRNA (Figure 6B) in the presence and absence of anti-IRES-oligo was determined. As expected, anti-IRES-oligo did not inhibit luciferase translation from bLuc mRNA (Figure 7, compare luciferase translation in lanes 3-4 to lanes 5-6) or CAT from bCRL (Figure 7, compare CAT translation in lanes 7-8 with lanes 9-10). Anti-IRES did however dramatically inhibit luciferase translation from bCRL mRNA (Figure 7, compare luciferase translation in lanes 7-8 with lanes 9-10). Thus, anti-IRES-oligo specifically inhibits rhinovirus IRES-dependent translation. In addition, modified nucleic acid or nucleic acid analogs as defined in Example 8a may also be utilized in the method of this example.

[0154] Luciferase activity assays were performed to quantitate the translational inhibition of luciferase from bL and bCRL mRNAs by anti-IRES-oligo. In agreement with the ^{35}S-methionine incorporation assay results, anti-IRES-oligo did not inhibit luciferase translation from bL mRNA (Figure 8, compare lanes 2 and 3) while it inhibited luciferase translation from bCRL mRNA approximately 95% (Figure 8, compare lanes 5 and 6). A control deoxyoligonucleotide (control-oligo, not shown) was synthesized with a reversed and complementary sequence to anti-IRES-oligo. The control deoxyoligonucleotide therefore contains approximately the same G-C and A-T composition, but cannot anneal nts 518-551 of the rhinovirus 14 IRES. Control-oligo had no effect on bL or bCRL mRNA translation (Figure 8, compare lane 4 with lane 2 and lane 7 with lane 5). Anti-IRES-oligo thus appears to specifically inhibit translation driven by the rhinovirus IRES.

Example 5: Reporter Gene Assays

CAT Spectrophotometric Assay

[0155] The most convenient technique for quantitating the rate of CM acetylation takes advantage of the generation of a free CoA sulfhydryl group coincident with transfer of the acetyl group to CM. Reaction of the reduced CoA with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) yields the mixed disulfide of CoA and thionitrobenzoic acid and a molar equivalent of free 5-thio-2-nitrobenzoate (Habeeb). The latter has a molar extinction coefficient of 13,600 at 412 nm. The assay is best carried out with a recording spectrophotometer equipped with a temperature-controlled cuvette chamber set at 37°C.

[0156] Reagents: Tris·hydrochloride, 1.0 M, pH 7.8, acetyl-CoA, 5 mM, chloramphenicol (D-*threo*) 5 mM, 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB). The only reagent solution that must be stored frozen in acetyl-CoA. The reaction mixture is freshly prepared from the individual reagents by dissolving 4 mg of DTNB in 1.0 ml of Tris-HCl buffer, after which 0.2 ml of the acetyl-CoA stock solution is added and the total volume is made up to 10 ml. The final concentrations of each component are as follows: Tris·HCl (100 mM), acetyl-CoA (0.1 mM), and DTNB (0.4 mg/ml). After the cuvette (1 cm light path) containing enzyme and the reaction mixture has been allowed to equilibrate with the waterbath, the reaction is started by the addition of CM at a final concentration of 0.1 mM. The rate of increase in absorption at 412 mM prior to the addition of CM is subtracted from the observed rate after the start of the reaction, and net change in extinction per minute is divided by 13.6 to give the result in micromoles per minute of CM-dependent DTNB reacted. Since the latter is equal to the rate of acetylation and since 1 unit of CAT = 1 μmole of CM acetylated per minute (37°C), the calculation also yields the number of units of enzyme in the cuvette.

[0157] An alternative spectrophotometric method can be used if a high concentration of competing mercaptans interferes with the DTNB assay. The loss of an acyl group from thioesters such as acetyl-CoA is accompanied by a decrease in absorption in the ultraviolet. The difference in molar extinction coefficients of acetyl-CoA and reduced CoA plus acetate is 4500 at 232 nm. Special care must be taken to remove interfering ultraviolet absorbing material from the enzyme preparation by gel filtration or dialysis. The contribution of the absorption due to protein added to the cuvette becomes a more serious obstacle in crude extracts, especially those with low levels of CAT activity. Apart from the inconvenience

of measurements in the far ultraviolet region and the fact that the method is intrinsically less sensitive than the DTNB procedure, the assay of thioester cleavage at 232 nm suffers from being a difference method. The absolute decreases in absorbance per unit time due to the presence of CM and low levels of CAT may be impossible to quantitate without recourse to the use of a dual beam recording spectrophotometer.

[0158] Radioisotopic CAT Assay: In this assay chloramphenicol acetyl transferase (CAT) transfers the ^{3}H-labeled acetyl group from acetyl CoA to chloramphenicol bound beads. The beads are washed and counted to determine CAT activity. This assay is approximately 2-5Y more sensitive than the spectrophotometric assay and will detect CAT in RRL. Materials: chloramphenicol-caproate-agarose (Sigma #C8899), [^{1}H] acetyl-CoA (Amersham TRK.688; specific activity >3Ci/mmol, 250 uCi/ml), acetyl-CoA (Sigma CO378; 100 mM in 50% DMSO (25 mg in 3.1 ml)), chloramphenicol (Sigma CO378; 100 mM in 50% DMSO), CAT (Sigma C8413),.10XTBS (50 mM Tris·HCl [pH 7.5], 150 mM NaCl), wash buffer: TBS containing 5 mM chloramphenicol and 1% SDS. Protocol: Thoroughly resuspend beads inside bottle and pipet 5 ml into Falcon tube. Rinse pipet with 8.5 mls H_2O and put in tube. Add 1.5 ml 10XTBS and spin 5 K rpm in Sorvall RC6000 rotor. Decant supernatant, refill tube with 1XTBS, respin, and decant supernatant. Add 1XTBS to 5 ml, and store excess beads at 4°C. To 100 μl rinsed beads and 2 μl substrate solution (15 mM cold acetyl CoA, 0.65 mM [^{3}H] acetyl CoA), add 2 μl CAT standard (dilutions 1:2 to 1:128 in TBS) or 5 μl translation reaction and incubate 20 minutes at 25°C. Add 1.25 ml wash buffer to quench reaction, then spin in centrifuge for 5 minutes at 14 K rpm. Carefully remove supernatant, leaving some liquid on beads. Repeat wash two more times, then add 100 μl H_2O and vortex. Immediately add scintillation fluid, cap, vortex upside down (to avoid clump of beads at bottom of tube which won't resuspend properly). Measure radioactivity in liquid scintillation spectrometer.

[0159] SEAP Assay: SEAP levels are determined by two distinct assays. The first assay measures the increase in light absorbance at 405 nm which accompanies the hydrolysis of *p*-nitrophenylphosphate (McComb and Bowers, 1972, Clin. Chem. 18, 97-104.). This assay is performed essentially as described in Example 16 above.

[0160] The bioluminescence-based assay for SEAP is performed essentially as described (Miska and Geiger, 1987, J. Clin. Chem. Clin. Biochem. 25, 23-30.). Fifty μl of freshly prepared substrate solution (0.1 mM D-luciferin-*O*-phosphate in LUPO buffer (10 mM diethanolamine, 0.5 mM $MgCl_2$, 10 mM L-homoarginine pH 9.8) and prewarmed to 37°C for 5 minutes in the dark. To this is added 50 μl of heated, clarified medium, prepared as described above, or a medium sample diluted in LUPO buffer. After a 30-minute incubation at 37°C in the dark, 100 μl of the reaction mixture are transferred into a tube containing 400 μl of bioluminescence buffer (30 mM Hepes pH 7.75, 5 mM $MgCl_2$, 0.66 mM EDTA, 0.1 mM DTT, 5 mM ATP) containing 1 μg (10^4 units) of luciferase. Light impulses are measured at 37°C in a luminometer (Berthold Biolumat, Model 9500T --10-s peak-measuring mode). All the chemicals used for the SEAP assays are obtained from Sigma (St. Louis, MO) except for luciferase, which is obtained from Boehringer-Mannheim (Indianapolis, IN) and D-luciferin-*O*-phosphate, which can be obtained from Novabiochem AG, CH-4448, Laufelfingen, Switzerland.

Example 6 : Cellular Assays

[0161] A dicistronic construct directing synthesis of two different reporter proteins is transfected into cells; cells are exposed to test compounds, then are tested for ability to produce reporter proteins. Production of both reporter proteins is preferably simultaneously or sequentially visualized or detected in same cell (luciferase, β-galactosidase).

A. Appropriate IRES-Reporter Gene Constructs

[0162] A monocistronic plasmid (pCMV-B-SEAP) and disistronic plasmid (pCMV-Luc-IRES-SEAP) are used to transfect cells and assay for translation in *vivo* in the presence and absence of test compounds. pCMV-B-SEAP contains, in order, the SV40 replication origin, cytomegalovirus (CMV) promoter, β-globin 5' nontranslated region, secreted alkaline phosphatase (SEAP) reporter gene, SV40 splice sites, and SV40 polyA signal. pCMV-Luc-IRES-SEAP contains, in order, the SV40 replication origin, cytomegalovirus (CMV) promoter, β-globin 5' nontranslated region, luciferase reporter gene, selected IRES element, SEAP reporter gene, SV40 splice sites, and SV40 polyA signal.

[0163] Test compounds are screened for their ability to inhibit SEAP synthesis driven by the IRES element from pB-luc-IRES-SEAP, but not inhibit luciferase synthesis dried by β-globin 5'NTR from pCMV-Luc-IRES-SEAP, but not inhibit luciferase synthesis driven by β-globin 5'NTR from pCMV-Luc-IRES-SEAP and not inhibit SEAP synthesis driven by β-globin 5'NTR from pCMV-B-SEAP. This screen selects test compounds which specifically inhibit translation from IRES elements without affecting normal cellular translation (from β-globin 5'NTR) or inhibiting SEAP activity.

[0164] IRES elements targeted include those from rhinovirus, coxsackievirus, poliovirus, echovirus, hepatitis A virus, hepatitis B virus, hepatitis C virus, mengo virus, encephalomycarditis virus, foot-and-mouth disease virus, theiler's murine encephalomyelitis virus, infectious bronchitis virus, vesicular stomatitis virus, and sendai virus.

B. Transfecting Cells with Dicistronic Plasmid

[0165] To denature DNA, mix DNA with 15 μl 20X HBSS (5.0 g Hepes, 8.0 g NaCl, 0.36 g KCl, 0.125 g Na_2HPO_4-H_2O, 1.0 g dextrose, H_2O to 50 ml), and bring up to 300 μl with H_2O, add 300 μl 1 mg/ml DAE dextran and incubate 4°C for 30 minutes. Grow COS1 cells on 6 cm plate to 50-70% confluent (100% confluent = complete), wash cells with 2 ml MEM media (+pen-strep, -serum) added to side of plate, tilt plate to cover cells, aspirate off medium by tipping plate and aspirating from side of plate. Repeat wash two more times. Transfect cells by adding 600 μl denatured DNA to cells at 25°C for 30 minutes with gentle rocking. Aspirate off dextran from cells, add 2 ml MEM (+2% fetal calf serum at 37°C) and incubate 37°C. To assay translation, prepare cell extract using Triton X-100 or freeze/thaw method and assay for SEAP and luciferase activity as described above.

Example 7 : Animal Model (s) of Picornavirus Infection

[0166] Described below are appropriate animal models which may be used to test potential drugs further. A model in which the infection is "exposed" such as a dermal, buccal, ocular or vaginal model is preferred.

A. Infection in Experimental Animals

[0167] A major characteristic of rhinoviruses is a high degree of species specificity. Chimpanzees have been infected with types 14 and 43 and gibbons with types 1A, 2, and 14; no overt illnesses were observed in the infected animals (Dick, 1968 Proc. Soc. Exp. Biol. Med. 127, 1079-1081; Pinto and Haff, 1969, Nature 224, 1310-1311). Inoculation of vervent and rhesus monkeys with M (monkey kidney grown) strains of virus did not produce infection. Infection was not produced in rabbits, guinea pigs, weanling mice, or 1-day-old mice injected with human rhinoviruses by the subcutaneous, intraperitoneal, or intravenous route similarly, intracranial injections of monkeys, hamsters, or baby mice did not induce either infection or disease (Hamparian et al., 1961, Proc Soc. Exp. Biol. Med. 108, 444-453; Jackson and Muldoon, 1973, J. Infect. Dis. 127, 328-355; Kisch et al., 1964, Am. J. Hyg. 79, 125-133). Intranasal inoculation of ferrets, hamsters, and newborn mice was also without effect. One of the animal rhinoviruses, equine rhinovirus, can infect other species including humans (Plummer, 1963, Arch. Ges. Virusforshchr 12 694-700.); a hamster model for use in screening of antiviral compounds has been developed that utilizes this virus. One of the human rhinoviruses, type 2, was recently adapted to grow in L cells (195); this virus was then used in a mouse model of rhinovirus infection where *in vitro* growth was demonstrated (196).

[0168] The cardioviruses (Columbia SK virus, EMC virus, ME virus, MM virus, and mengovirus) all belong to a single serotype and are here all considered to be strains of EMC virus. They are generally regarded as murine viruses although their host range includes humans, pigs, elephants, and squirrels among others.

[0169] The Theiler's murine encephalomyelitis viruses (TMEV), also representing a single serotype, are divided into two groups, typified by strains called GDVII and TO. the GDVII group causes an acute polio-like disease in mice. The TO group are less virulent and cause a chronic demyelinating disease resembling multiple sclerosis and have thus become important models for study of this and other motor neuron diseases (Lipton and Rozhan, 1986, Bhatt, ed., Viral and Mycoplasma Infection of Laboratory Rodents, pp. 253-276, Academic Press, Orlando.217).

[0170] Apthoviruses (foot-and-mouth disease viruses) infect cloven-footed animals, especially cattle, goats, pigs, sheep, and, rarely, even humans.

[0171] Some picornaviruses, such as cricket paralysis virus (Tinsley et al., 1984, Intervirology 21, 181-186.) infect insects (Longworth, 1978, Adv. Virus Res. 23, 103-157.; Moore and Tinsley, 1982, Arch. Virol. 72, 229-245.; Scotti et al., 1981, Adv. Virus Res. 26, 117-142.).

B. Experimental Infection, Host Range

[0172] The host range of the enteroviruses varies greatly from one type to the next and even among strains of the same type. They may readily be induced, by laboratory manipulation, to yield variants that have host ranges and tissue tropisms different from those of wild strains; this has led to the development of attenuated poliovaccine strains.

[0173] Polioviruses have a very restricted host range among laboratory animals (Bodian, 1959, In: Rivers and Horsfall, eds., Viral and Rickettsial Infections of Man, Third ed., pp. 430-473, 479-518, Lippincott, Philadelphia). Most strains will infect and cause flaccid paralysis only in monkeys and chimpanzees. Infection is initiated most readily by direct inoculation into the brain or spinal cord. Chimpanzees and cynomolgus monkeys can also be infected by the oral route; in chimpanzees, the infection thus produced is usually asymptomatic. The animals become intestinal carriers of the virus; they also develop a viremia that is quenched by the appearance of antibodies in the circulating blood. Unusual strains have been transmitted to mice or chick embryos.

[0174] The original criteria for classification as a member of the echovirus group included the provision that the prototype

strains fail to produce disease in suckling mice or in monkeys. However, different strains can produce variants that exhibit animal pathogenicity. A number of echoviruses have produced inapparent infections in monkeys, with mild lesions in the CNS (Wenner, 1962, Ann NY Acad. sci. 101, 398-412.). In the chimpanzee, no apparent illness is produced, but infection can be demonstrated by the presence and persistence of virus in the throat and in the feces and by type-specific antibody responses (Itoh and Melnick, 1957, J. Exp. Med. 106, 677-688.). Initially, echoviruses were distinguished from coxsackieviruses by their failure to produce pathological changes in newborn mice; this led to the early classification of these strains as coxsackievirus A23. Conversely, strains of some coxsackievirus types (especially A9) lack mouse pathogenicity and thus resemble echoviruses. This variability in biological properties is the chief reason why new members of the genus are no longer being sub-classified as echoviruses or coxsackieviruses but are simply called enteroviruses.

[0175] The cardinal feature of coxsackieviruses is their infectivity for newborn mice (Daldorf and Melnick, 1965, In: Horsfall and Tamm, eds., Viral and Rickettsial Infections of Man, Fourth ed., pp. 474-512, Lippincott, Philadelphia). Chimpanzees and cynomolgus monkeys can be infected subclinically; virus appears in the blood and throat for short periods and is excreted in the feces for 2-5 weeks. Type A14 produces poliomyelitis-like lesions in adult mice and in monkeys, but in suckling mice this type produces only myositis. Type A7 strains produce paralysis and severe CNS lesions in monkeys (Dalldorf, 1957, J. Exp. Med. 106, 69-76.;,268), and at one time this serotype was considered to be a fourth type of poliovirus.

[0176] Group A coxsackieviruses characteristically produce widespread myositis in the skeletal muscles of newborn mice, resulting in flaccid paralysis without other observable lesions (Daldorf and Melnick, 1965, In: Horsfall and Tamm, eds., Viral and Rickettsial Infections of Man, Fourth ed., pp. 474-512, Lippincott, Philadelphia). In addition to being able to infect the immature skeletal muscles of newborn mice, coxsackieviruses of the A group also can infect surgically denervated muscles of adult mice, whereas mature innervated muscles are relatively resistant. Leg muscles of adult mice in which quantal release of acetylcholine had been blocked with botulinum toxin were susceptible when subsequently injected with coxsackievirus A2 (Andrew et al., 1984, Science 223, 714-716.). Since the only known action of the toxin is the effect on acetylcholine release, the findings suggest that synaptic transmission has a role in preventing the susceptibility of skeletal muscles to coxsackievirus infection.

[0177] Group B viruses can produce a myositis that is more focal in distribution than that produced by viruses of group A, but they also give rise to a necrotizing steatitis involving principally the natural fetal fat lobules (e.g., intrascapular pads, cervical and cephalic pads). Encephalitis is found at times; the animals die with paralysis of the spastic type. Some B strains also produce pancreatitis, myocarditis, endocarditis, and hepatitis in both suckling and adult mice. The corticosteroids may enhance the susceptibility of older mice to infection of the pancreas. Normal adult mice tolerate infections with group B coxsackieviruses, but in mice subjected to sustained postweaning undernutrition (marasmus), coxsackievirus B3 produces severe disease, including persistence of infective virus in the heart, spleen, liver, and pancreas. Lymphoid tissues are markedly atrophic in marasmic animals. Transfer of lymphoid cells from normal mice immunized against the virus provides virus-infected marasmic mice with significant protection against severe sequelae (Woodruff and Woodruff, 1971, Proc. Natl. Acad. Sci. USA 68, 2108-2111). These observations support the hypothesis that lymphocyte-mediated defense mechanisms may play an important role in normal recovery from primary viral infections (Paque, 1981, Infect. Immun. 31, 470-479.; Woodruff, 1980, Am J. Pathol. 101, 427-478.205, 283). Athymic mice exposed to coxsackievirus B3 develop a persistent infection in which the myocardium is affected in a disseminated, multifocal way. The RNA viral genome can readily be detected in the myocardium by the use of radioactively labeled cloned coxsackie B3 cDNA (Kanbdolf et al., 1987, Proc. Natl. Acad. Sci. USA 84, 6272-6276).

Administration of agents

[0178] In practicing the methods of the invention, the compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These compositions can be utilized in vivo, ordinarily in a mammal, preferably in a human, or in vitro. In employing them in vivo, the compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, vaginally, nasally, orally, transdermally, topically, ocularly, intraperitoneally, or as suitably formulated surgical implants employing a variety of dosage forms. As will be readily apparent to one skilled in the art, the useful in vivo dosage to be administered and the particular mode of administration will vary depending upon the mammalian species treated, the particular composition employed, and the specific use for which these compositions are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art. Typically, applications of compositions are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved.

[0179] The dosage for the compositions of the present invention can range broadly depending upon the desired affects and the therapeutic indication. Typically, dosages will be between about 0.01 μg and 100 mg/kg, preferably between about 0.01 and 10 mg/kg, body weight. Administration is preferably *per os* on a daily or as-needed basis.

[0180] Orally-administered formulations can be prepared in conventional forms, including capsules, chewable tablets,

enteric-coated tablets, syrups, emulsions, suspensions, or as solid forms suitable for solution or suspension in liquid prior to administration. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride or the like. In addition, if desired, the pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (e.g., liposomes) may be utilized.

[0181] In selected cases, drug delivery vehicles may be employed for systemic or topical administration. They can be designed to serve as a slow release reservoir, or to deliver their contents directly to the target cell. An advantage of using direct delivery drug vehicles is that multiple molecules are delivered per vehicle uptake event. Such vehicles have been shown to also increase the circulation half-life of drugs which would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, biodegradable polymers (surgical implants or nanocapsules), and bioadhesive microspheres.

[0182] For example, a liposome delivery vehicle originally designed as a research tool, Lipofectin, has been shown to deliver intact molecules to cells. Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost-effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of' this technology as an acceptable drug delivery system.

[0183] Other controlled release drug delivery systems, such as nanoparticles and hydrogels may be potential delivery vehicles for an agent. These carriers have been developed for chemotherapeutic agents.

[0184] Topical administration of agents is advantageous since it allows localized concentration at the site of administration with minimal systemic adsorption. This simplifies the delivery strategy of the agent to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be administered is far less than that required for other administration routes.

[0185] Effective delivery requires the agent to diffuse into the infected cells. Chemical modification of the agent may be all that is required for penetration. However, in the event that such modification is insufficient, the modified agent can be co-formulated with permeability enhancers, such as Azone or oleic acid, in a liposome. The liposomes can either represent a slow release presentation vehicle in which the modified agent and permeability enhancer transfer from the liposome into the infected cell, or the liposome phospholipids can participate directly with the modified agent and permeability enhancer in facilitating cellular delivery.

[0186] Agents may also be systemically administered. Systemic absorption refers to the accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: oral, intravenous, subcutaneous, intraperitoneal, intranasal, intrathecal and ocular. Each of these administration routes exposes the agent to an accessible diseased tissue. Subcutaneous administration drains into a localized lymph node which proceeds through the lymphatic network into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier can localize the agent at the lymph node and participate in the delivery of the agent to the cell.

[0187] A formulation which can associate agents with the surface of lymphocytes and macrophages is also useful. This will provide enhanced delivery to, for example, HSV-infected cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of infected cells.

[0188] Intraperitoneal administration also leads to entry into the circulation with the molecular weight or size of the agent-delivery vehicle complex controlling the rate of entry.

[0189] Liposomes injected intravenously show accumulation in the liver, lung and spleen. The composition and size can be adjusted so that this accumulation represents 30% to 40% of the injected dose. The rest is left to circulate in the blood stream for up to 24 hours.

[0190] All publications referenced herein are hereby incorporated by reference herein, including the nucleic acid sequences listed in each publication.

[0191] Other embodiments are within the following claims.

SEQUENCE LISTING

[0192]

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: METHOD FOR SELECTIVE INACTIVATION OF VIRAL REPLICATION

(iii) NUMBER OF SEQUENCES: 26

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/042,024
(B) FILING DATE: 02-APR-1993

(viii) ATTORNEY/AGENT INFORMATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
ATAGGTACCT AATACGACTC ACTATAGGGA CACTTGCTTT TGACAC 46

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
ATAGGGCCCC TCGAGGTCTG TTTTGGGGG 29

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
ATAGGATCCT TAAAACAGCG GATGGG 26

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
GGCGTCTTCC ATGATCACAG 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
ATAGGGCCCT GATCATGCTG CTGCTGCTGC TGC 33

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
ATAGTCGACT TAACCCGGGT GCGCGGCG 28

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
ATACTCGAGA TGGAAGACGC CAAAAAC 27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
ATACCTAGGT TACAATTTGG ACTTTCCGC 29

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
ATAGGTACCT AATACGACTC ACTATAGGGA CACTTGCTTT TGACAC 46

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs
(B) TYPE : nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
ATAGGGCCCC TCGAGGTCTG TTTTGGGGG 29

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
ATAGGGCCCT GATCATGGAA GACGCCAAAA AC 32

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

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

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
ATAGTCGACT TACAATTTGG ACTTTCCGC 29

(2) INFORMATION FOR SEQ ID NO : 13 :

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
ATACTCGAGA TGAGCTTGGC GAGATTTTCA GG 32

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
ATACCTAGGT TACGCCCCGC CCTGCC 26

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
ATAGGATCCT TAAAACAGCG GATGGG 26

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:
GGCGTCTTCC ATGATCACAG 20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1515 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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

ATGGTGGCCC CCGGCTCTGT GACCAGCCGG CTGGGCTCGG TGTTCCCTTT CCTGCTGGTC 60

CTGGTGGACC TGCAGTACGA AGGTGCTGAA TGTGGAGTAA ATGCAGATGT TGAGAAGCAT 120

CTGGAATTGG GCAAGAAGCT GCTCGCAGCC GGACAGCTCG CGGATGCGTT ATCTCAGTTT 180

CACGCTGCAG TAGATGGTGA CCCTGATAAC TATATTGCTT ACTATCGGAG AGCTACTGTC 240

TTTTTAGCTA TGGGCAAATC AAAAGCAGCA CTTCCTGATT TAACTAAAGT GATTGAATTG 300

AAGATGGATT TCACTGCAGC AAGATTACAG AGAGGTCACT TATTACTCAA ACAAGGAAAA 360

CTTGATGAAG CAGAAGATGA TTTTAAAAAA GTGCTCAAGT CAAATCCAAG TGAAAATGAA 420

GAGAAGGAGG CCCAGTCCCA GCTTGTCAAA TCTGATGAAA TGCAGCGTCT GCGCTCACAA 480

GCACTGGATG CCTTTGAGAG CTCAGATTTT ACTGCTGCTA TAACCTTCCT TGATAAGATT 540

TTAGAGGTTT GTGTTTGGGA TGCAGAACTT CGAGAACTTC GAGCTGAATG TTTTATAAAA 600

GAAGGGGAAC CTAGGAAAGC GATAAGTGAC TTAAAAGCTT CATCAAAATT GAAAAACGAT 660

AATACTGAGG CATTTTATAA AATCAGCACA CTCTACTATG AACTAGGAGA CCATGAACTG 720

TCTCTCAGTG AAGTTCGTGA ATGTCTTAAA CTTGACCAGG ATCATAAAAG GTGTTTTGCA 780

CACTATAAAC AAGTAAAGAA ACTGAATAAG CTGATTGAGT CAGCTGAAGA GCTCATCAAA 840

GAAGGCAGGT ACACAGATGC AATCAGCAAA TATGAATCTG TCATGAAAAC AGAGCCAGGT 900

GTTCATGAAT ATACAATTCG TTCAAAAGAA AGGATTTGCC ACTGCTTTTC TAAGGATGAG 960

AAGCCTGTTG AAGCTATTCG AGTATGTTCA GAAGTTTTAC AGGTGGAACC TGACAACGTG 1020

AATGCTCTGA AAGACCGAGC AGAGGCCTAT TTAATAGAAG AAATGTATGA TGAAGCTATT 1080

CAGGATTATG AAACTGCTCA GGAACACAAT GAGAATGATC AGCAGATTCG AGAAGGTCTG 1140

GAGAAAGCAC AGAGGCTACT GAAACAGTCA CAGAGACGAG ATTATTACAA AATCTTGGGA 1200

GTAAAAAGAA ATGCCAAAAA GCAAGAAATC ATTAAAGCAT ACCGAAAATT AGCACTGCAG 1260

TGGCACCCAG ACAACTTCCA GAACGAAGAA GAAAAGAAAA AAGCTGAGAA GAAGTTCATT 1320

GACATAGCAG CTGCTAAAGA AGTCCTCTCC GATCCAGAAA TGAGGAAGAA GTTTGATGAC 1380

GGAGAAGACC CCCTGGACGC AGAGAGCCAA CAAGGAGGTG GCGGCAACCC TTTCCACAGG 1440

AGCTGGAACT CATGGCAAGG GTTCAGTCCC TTTAGCTCAG GCGGACCTTT TAGATTTAAA 1500

TTCCACTTCA ATTAA 1515

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:
AATAGAATTC TAATACGACT CACTATAGGG ACACTTGCTT TTGACAC 47

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:
ATAAGGTACC TCTGTCTGTT TTGGGGG 27

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:
AATACTGCAG TGATCATGGA AGACGCCAAA AACATAAAG 39

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
AATAAAGCTT GGGCCCTTAC AATTTGGACT TTCCGC 36

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:
AATAGGTACC ATGGAGAAAA AAATCACTGG ATATACC 37

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:
AATAGGATCC TTACGCCCCG CCCTGCC 27

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:
AATAGGATCC TTAAAACAGC GGATGGG 27

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:
AAAACTGCAG CATGCTGATC ACAGTATATG TATATATATG CTGTGACC 48

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:
AGTAGTCGGT CCCGTCCCGG AATTGCGCAT TACG 34

Claims

1. A method of screening for an agent that inhibits translation initiation from a viral IRES comprising:

(a) contacting a test agent with a cell comprising a recombinant nucleic acid comprising:

a viral internal ribosome entry site (IRES); and
a polynucleotide encoding a reporter protein,

wherein said IRES is operably linked to said polynucleotide;
(b) assessing, if present, any production of said report protein in the presence of the test agent; and
(c) comparing the level of production of the reporter protein in the presence of the test agent with the level of production of the reporter protein in the absence of the test agent;

wherein a reduction of the reporter protein in the presence of the test agent indicates that the test agent is an agent that inhibits translation initiation from the viral IRES.

2. The method of claim 1, wherein said recombinant nucleic acid further comprises:

a eukaryotic mRNA-5'-terminal cap and an untranslated (UTR) region upstream of said IRES, and downstream of said cap and UTR region a second reporter protein.

3. The method of claim 1 or 2, wherein said IRES is a Hepatitis A IRES

4. The method of claim 1 or 2, wherein said IRES is a Hepatitis C IRES.

5. The method of claim 1 or 2, wherein said IRES is an HIV IRES,

6. The method of any one of claims 1 to 5, wherein said recombinant nucleic acid is an RNA.

7. The method of any one of claims 1 to 6, wherein said amount of said reporter protein is determined by assessing a signal produced by said reporter protein.

8. The method of claim 1, wherein said IRES is an IRES from a rhinovirus, coxsackievirus, poliovirus, echovirus, hepatitis A virus, hepatitis B virus, hepatitis C virus, mengo virus, encephalomyocarditis virus, foot and mouth disease

virus, Theiler's murine encephalomyelitis virus, infections bronchitis virus, vesicular stomatitis virus or sendai virus.

9. The method of claim 1, wherein the IRES is an IRES from a picornavirus, hepatitis virus A, hepatitis virus B, hepatitis virus C, influenza virus, HIV, herpes virus or cytomegalovirus.

10. The method of claim 1, wherein said IRES is a picornavirus IRES.

11. Use of a cell comprising a recombinant nucleic acid comprising:

a viral internal ribosome entry site (IRES); and
a polynucleotide encoding a reporter protein,

wherein said IRES is operably linked to said polynucleotide ; for determining whether a test agent inhibits translation initiation from the viral IRES.

12. The use of claim 11, wherein said recombinant nucleic acid further comprises:

a eukaryotic mRNA-5'-terminal cap and an untranslated (UTR) region upstream of said IRES, and downstream of said cap and UTR region a second reporter protein.

13. The use of claim 11 or 12, wherein said IRES is a Hepatitis A IRES.

14. The use of claim 11 or 12, wherein said IRES is a Hepatitis C IRES.

15. The use of claim 11 or 12, wherein said IRES is an HIV IRES.

16. The use of any one of claims 11 to 15, wherein said recombinant nucleic acid is an RNA.

17. The use of any of claims 11-16, wherein said amount of said reporter protein is determined by assessing a signal produced by said reporter protein.

18. The use of claim 11, wherein said IRES is an IRES from a rhinovirus, coxsackievirus, poliovirus, echovirus, hepatitis A virus, hepatitis B virus, hepatitis C virus, mengo virus, encephalomyocarditis virus, foot and mouth disease virus, Theiler's murine encephalomyelitis virus, infections bronchitis virus, vesicular stomatitis virus or sendai virus.

19. A cell for screening for an agent that inhibits translation initiation from a viral IRES, the cell comprising a recombinant nucleic acid comprising:

a viral internal ribosome entry site (IRES); and
a polynucleotide encoding a protein that confers a growth disadvantage on said cell,

wherein said viral IRES is operably linked to said polynucleotide.

20. The cell of claim 19, wherein said recombinant nucleic acid further comprises:

a eukaryotic mRNA-5'-tenninal cap and an untranslated (UTR) region upstream of said IRES, and downstream of said cap and UTR a second reporter protein.

21. The cell of claim 19 or 20, wherein said IRES is an IRES from a rhinovirus, coxsackievirus, poliovirus, echovirus, hepatitis A virus, hepatitis B virus, hepatitis C virus, mengo virus, encephalomyocarditis virus, foot and mouth disease virus, Theiler's murine encephalomyelitis virus, infections bronchitis virus, vesicular stomatitis virus or sendai virus.

22. The cell of claim 19 or 20, wherein said IRES is an IRES from a picornavirus, hepatitis virus A, hepatitis virus B, hepatitis virus C, influenza virus, HIV, herpes virus or cytomegalovirus.

23. The cell of claim 19 or 20, wherein said IRES is a picornavirus IRES.

24. The cell of claim 19 or 20, wherein said IRES is a hepatitis C virus IRES.

25. The cell of claim 19 or 20, wherein said IRES is an HIV IRES.

26. The cell of any one of claims 19-25, wherein said cell is a mammalian cell.

27. The cell of claim 19 or 20, wherein said protein is an enzyme that converts a non-toxic substrate into a toxic product,

28. The cell of claim 19 or 20, wherein said protein is toxic to said cell.

Patentansprüche

1. Verfahren zum Screenen auf ein Mittel, das den Translationsbeginn aus einer viralen IRES hemmt, das Folgendes umfasst;

(a) Kontaktieren eines Testmittels mit einer Zelle, die eine rekombinante Nucleinsäure umfasst, die
eine virale interne Ribosomen-Eintrittstelle (IRES); und
ein Polynucleotid, das für ein Reporterprotein kodiert, umfasst,
worin die IRES operabel an das Polynucleotid gebunden ist;
(b) Beurteilung, wenn vorhanden, der Produktion des Reporterproteins in Gegenwart eines Testmittels; und
(c) Vergleich des Produktionsausmaßes des Reporterproteins in Gegenwart des Testmittels mit dem Produktionsausmaß des Reporterproteins in Abwesenheit des Testmittels,

worin eine Reduktion des Reporterproteins in Gegenwart des Testmittels angibt, dass das Testmittel ein Mittel ist, welches den Translationsbeginn aus der viralen IRES hemmt.

2. Verfahren nach Anspruch 1, worin die rekombinante Nucleinsäure weiters Folgendes umfasst:

eine 5'-terminale eukaryotische mRNA-Cap und eine nichttranslatierte (UTR-) Region stromauf der IRES und ein zweites Reporterprotein stromab der Cap und UTR-Region.

3. Verfahren nach Anspruch 1 oder 2, worin die IRES eine Hepatitis-A-IRES ist.

4. Verfahren nach Anspruch 1 oder 2, worin die IRES eine Hepatitis-C-IRES ist.

5. Verfahren nach Anspruch 1 oder 2, worin die IRES eine HIV-IRES ist.

6. Verfahren nach einem der Ansprüche 1 bis 5, worin die rekombinante Nucleinsäure eine RNA ist.

7. Verfahren nach einem der Ansprüche 1 bis 6, worin die Menge des Reporterproteins durch die Beurteilung eines vom Reporterprotein produzierten Signals bestimmt wird.

8. Verfahren nach Anspruch 1, worin die IRES eine IRES aus einem Rhinovirus, Coxsackie-Virus, Poliomyelitisvirus, ECHO-Virus, Hepatitis-A-Virus, Hepatitis-B-Virus, Hepatitis-C-Virus, Mengovirus, Enzephalomyokarditis-Virus, Maul-und-Klauenseuchen-Virus, einem murinen Enzephalomyelitisvirus von Theiler, einem infektiösen Bronchitisvirus, einem Gingivostomatitis-herpetica-Virus oder einem Sendai-Virus ist.

9. Verfahren nach Anspruch 1, worin die IRES eine IRES aus einem Picornavirus, Hepatitis-A-Virus, Hepatitis-B-Virus, Hepatitis-C-Virus, Influenzavirus, aus HIV, aus einem Herpes-Virus oder Cytomegalievirus ist.

10. Verfahren nach Anspruch 1, worin die IRES eine Picornavirus-IRES ist.

11. Verwendung einer Zelle, umfassend eine rekombinante Nucleinsäure, die
eine virale interne Ribosomen-Eintrittstelle (IRES); und
ein Polynucleotid, das für ein Reporterprotein kodiert, umfasst,
worin die IRES operabel an das Polynucleotid gebunden ist;
zur Bestimmung, ob ein Testmittel den Translationsbeginn aus der viralen IRES hemmt.

12. Verwendung nach Anspruch 11, worin die rekombinante Nucleinsäure weiters Folgendes umfasst:

eine 5'-terminale eukaryotische mRNA-Cap und eine nichttranslatierte (UTR-) Region stromab der IRES und ein zweites Reporterprotein stromab der Cap und der UTR-Region.

13. Verwendung nach Anspruch 11 oder 12, worin die IRES eine Hepatitis-A-IRES ist.

14. Verwendung nach Anspruch 11 oder 12, worin die IRES eine Hepatitis-C-IRES ist.

15. Verwendung nach Anspruch 11 oder 12, worin die IRES eine HIV-IRES ist.

16. Verwendung nach einem der Ansprüche 11 bis 15, worin die rekombinante Nucleinsäure eine RNA ist.

17. Verwendung nach einem der Ansprüche 11-16, worin die Menge des Reporterproteins durch Beurteilung eines vom Reporterprotein produzierten Signals bestimmt wird.

18. Verwendung nach Anspruch 11, worin die IRES eine IRES aus einem Rhinovirus, Coxsackie-Virus, Poliomyelitisvirus, ECHO-Virus, Hepatitis-A-Virus, Hepatitis-B-Virus, Hepatitis-C-Virus, Mengovirus, Enzephalomyokarditis-Virus, Maul-und-Klauenseuchen-Virus, einem murinen Enzephalomyelitisvirus von Theiler, einem infektiösen Bronchitisvirus, einem Gingivostomatitis-herpetica-Virus oder einem Sendai-Virus ist.

19. Zelle zum Screenen auf ein Mittel, das den Translationsbeginn aus einer viralen IRES hemmt, worin die Zelle eine rekombinante Nucleinsäure umfasst, die
eine virale interne Ribosomen-Eintrittstelle (IRES); und
ein Polynucleotid, das für ein Protein kodiert, das dieser Zelle einen Wachstumsnachteil verleiht, umfasst,
worin die virale IRES operabel an das Polynucleotid gebunden ist.

20. Zelle nach Anspruch 19, worin die rekombinante Nucleinsäure weiters Folgendes umfasst:

eine 5'-terminale eukaryotische mRNA-Cap und eine nichttranslatierte (UTR-) Region stromauf der IRES und ein zweites Reporterprotein stromab der Cap und UTR.

21. Zelle nach Anspruch 19 oder 20, worin die IRES eine IRES aus einem Rhinovirus, Coxsackie-Virus, Poliomyelitisvirus, ECHO-Virus, Hepatitis-A-Virus, Hepatitis-B-Virus, Hepatitis-C-Virus, Mengovirus, Enzephalomyokarditis-Virus, Maul-und-Klauenseuchen-Virus, einem murinen Enzephalomyelitisvirus von Theiler, einem infektiösen Bronchitisvirus, einem Gingivostomatitis-herpetica-Virus oder einem Sendai-Virus ist.

22. Zelle nach Anspruch 19 oder 20, worin die IRES eine IRES von einem Picornavirus, Hepatitis-A-Virus, Hepatitis-B-Virus, Hepatitis-C-Virus, Influenzavirus, von HIV, einem Herpes-Virus oder Cytomegalievirus ist.

23. Zelle nach Anspruch 19 oder 20, worin die IRES eine Picornavirus-IRES ist.

24. Zelle nach Anspruch 19 oder 20, worin die IRES eine Hepatitis-C-Virus-IRES ist.

25. Zelle nach Anspruch 19 oder 20, worin die IRES eine HIV-IRES ist.

26. Zelle nach einem der Ansprüche 19-25, worin die Zelle eine Säugetierzelle ist.

27. Zelle nach einem der Ansprüche 19 oder 20, worin das Protein ein Enzym ist, das ein nichttoxisches Substrat in ein toxisches Produkt konvertiert.

28. Zelle nach einem der Ansprüche 19 oder 20, worin das Protein für die Zelle toxisch ist.

Revendications

1. Méthode de criblage d'un agent qui inhibe l'initiation de traduction à partir d'un IRES viral comprenant:

(a) la mise en contact d'un agent de test avec une cellule comprenant un acide nucléique recombinant comprenant:

un site d'entrée de ribosome interne viral (IRES); et
un polynucléotide codant pour une protéine reporteur,
où ledit IRES est opérativement enchaîné audit polynucléotide; et
(b) l'évaluation, si elle est présente, de toute production de ladite protéine reporteur en présence de l'agent de test; et
(c) la comparaison du niveau de production de la protéine reporteur en présence de l'agent de test avec le niveau de production de la protéine reporteur en l'absence de l'agent de test;

où une réduction de la protéine reporteur en présence de l'agent de test indique que l'agent de test est un agent qui inhibe l'initiation de traduction à partir de IRES viral.

2. Méthode de la revendication 1, où ledit acide nucléique recombinant comprend de plus:

un capuchon 5'-terminal d'ARNm eucaryote et une région non traduite (UTR) en amont dudit IRES, et en aval dudit capuchon, une région UTR d'une seconde protéine reporteur.

3. Méthode de la revendication 1 ou 2, où ledit IRES est un IRES de l'Hépatite A.

4. Méthode de la revendication 1 ou 2, où ledit IRES est un IRES de l'Hépatite C.

5. Méthode de la revendicatioin 1 ou 2, où ledit IRES est un IRES de VIH.

6. Méthode de l'une quelconque des revendications 1 à 5, où ledit acide nucléique recombinant est un ARN.

7. Méthode de l'une quelconque des revendications 1 à 6, où ladite quantité de ladite protéine reporteur est déterminée en évaluant un signal produit par ladite protéine reporteur.

8. Méthode de la revendication 1, où ledit IRES est un IRES d'un rhinovirus, d'un coxsackievirus, d'un poliovirus, d'un échovirus, d'un virus de l'hépatite A, d'un virus de l'hépatite B, d'un virus de l'hépatite C, d'un virus de mengo, d'un virus d'encéphalomyocardite, d'un virus de la maladie du pied et de la bouche, d'un virus de l'encéphalomyélite du muridé de Theiler, d'un virus d'infections de bronchite, d'un virus de stomatite vésiculaire ou d'un virus de sendai.

9. Méthode de la revendication 1, où IRES est IRES d'un picornavirus, d'un virus de l'hépatite A, d'un virus de l'hépatite B, d'un virus de l'hépatite C, d'un virus de la grippe, d'un virus de VIH, d'un virus de l'herpès ou d'un cytomégalovirus.

10. Méthode de la revendication 1, où ledit IRES est un IRES de picornavirus.

11. Utilisation d'une cellule comprenant un acide nucléique recombinant comprenant:

un site d'entrée de ribosome viral interne (IRES); et
un polynucléotide codant pour une protéine reporteur,

où ledit IRES est opérativement enchaîné audit polynucléotide;
pour déterminer si un agent de test inhibe l'initiation de traduction du IRES viral.

12. Utilisation de la revendication 11, où ledit acide nucléique recombinant comprend de plus:

un capuchon 5'-terminal d'ARNm et une région non traduite (UTR) en amont dudit IRES, et en aval dudit capuchon et de ladite région UTR, une seconde protéine reporteur.

13. Utilisation de la revendicationi 12, où ledit IRES est un IRES de l'Hépatite A.

14. Utilisation de la revendication 11 ou 12, où ledit IRES est un IRES de l'Hépatite C.

15. Utilisation de la revendication 11 ou 12, où ledit IRES est un IRES de VIH.

16. Utilisation de l'une quelconque des revendications 11 à 15, où ledit acide nucléique recombinant est un ARN.

17. Utilisation de l'une quelconque des revendications 11-16, où ladite quantité de ladite protéine reporteur est déterminée en évaluant un signal produit par ladite protéine reporteur.

18. Utilisation de la revendication 11, où ledit IRES est un IRES d'un rhinovirus, coxsackievirus, poliovirus, échovirus, virus de l'hépatite A, virus de l'hépatite B, virus de l'hépatite C, virus de mengo, virus de l'encéphalomyocardite, virus de la maladie de pied et la bouche, virus de l'encéphalomyélite de muridé de Theiler, virus d'infections de bronchite, virus de la stomatite vésiculaire ou virus de sendai.

19. Cellule pour cribler un agent qui inhibe l'initiation de traduction à partir d'un IRES, la cellule comprenant un acide nucléique recombinant comprenant:

un site d'entrée de ribosome interne viral (IRES); et
un polynucléotide codant pour une protéine qui confère un désavantage de croissance à ladite cellule,

où ledit IRES viral est opérativement enchaîné audit polynucléotide.

20. Cellule de la revendication 19, où ledit acide nucléique recombinant comprend de plus:

un capuchon 5'-terminal d'ARNm eucaryote et une région non traduite (UTR) en amont dudit IRES, et en aval dudit capuchon et de UTR, une seconde protéine reporteur.

21. Cellule de la revendication 19 ou 20, où ledit IRES est un IRES d'un rhinovirus, coxsackievirus, poliovirus, échovirus, virus de l'hépatite A, virus de l'hépatite B, virus de l'hépatite C, virus de mengo, virus de l'encéphalomyocardite, virus de la maladie de pied et la bouche, virus de l'encéphalomyélite de muridé de Theiler, virus d'infections de bronchite, virus de la stomatite vésiculaire ou virus de sendai.

22. Cellule de la revendication 19 ou 20, où ledit IRES est un IRES d'un picornavirus, d'un virus de l'hépatite A, d'un virus de l'hépatite B, d'un virus de l'hépatite C, d'un virus de la grippe, de VIH, du virus de l'herpès ou du cytomégalovirus.

23. Cellule de la revendication 19 ou 20, où ledit IREs est un IRES de picornavirus.

24. Cellule de la revendication 19 ou 20, où ledit IRES est un IRES de virus de l'hépatite C.

25. Cellule de la revendication 19 ou 20, où ledit IRES est un IRES de VIH.

26. Cellule de l'une quelconque des revendications 19-25, où ladite cellule est un cellule mammalienne.

27. Cellule de la revendication 19 ou 20, où ladite protéine est une enzyme qui convertit un substrat non toxique en un produit toxique.

28. Cellule de la revendication 19 ou 20, où ladite protéine est toxique vis-à-vis de ladite cellule.

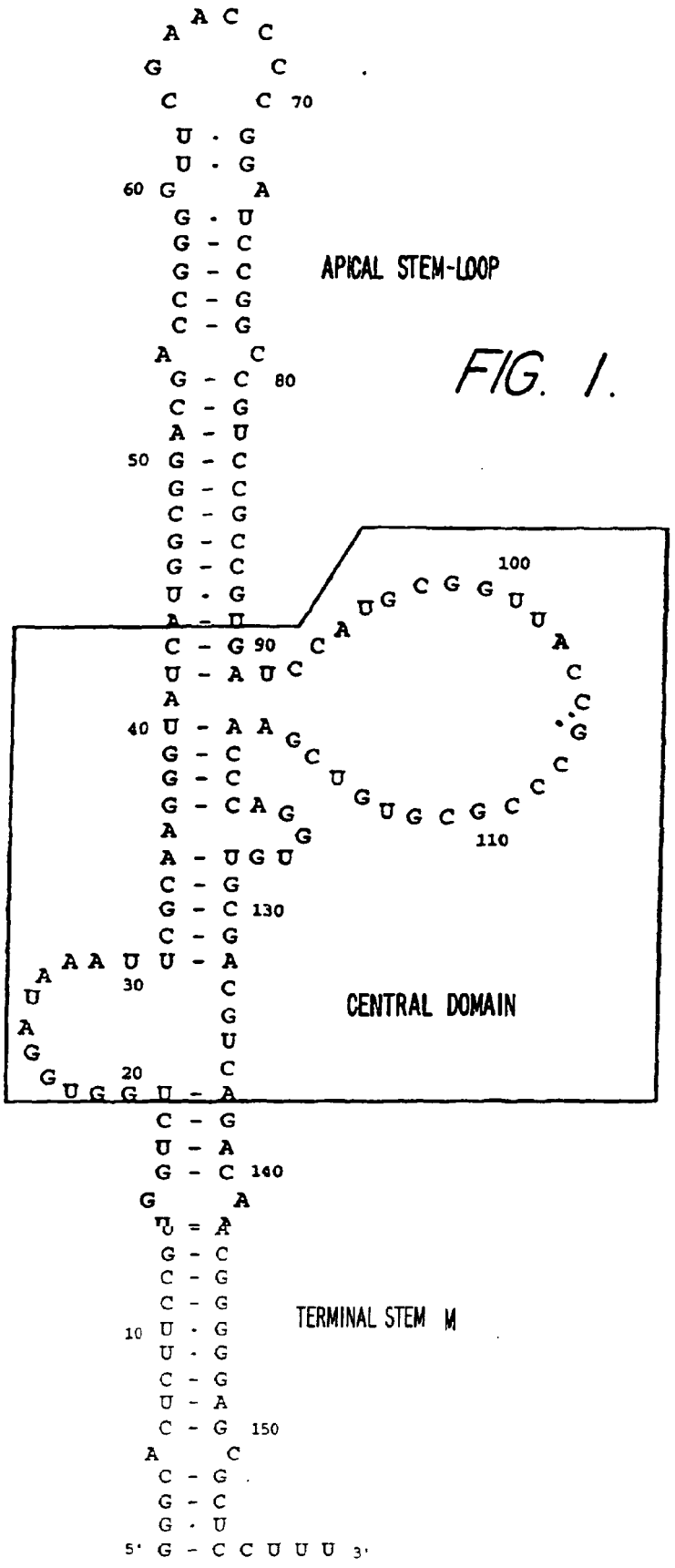

FIG. 1.

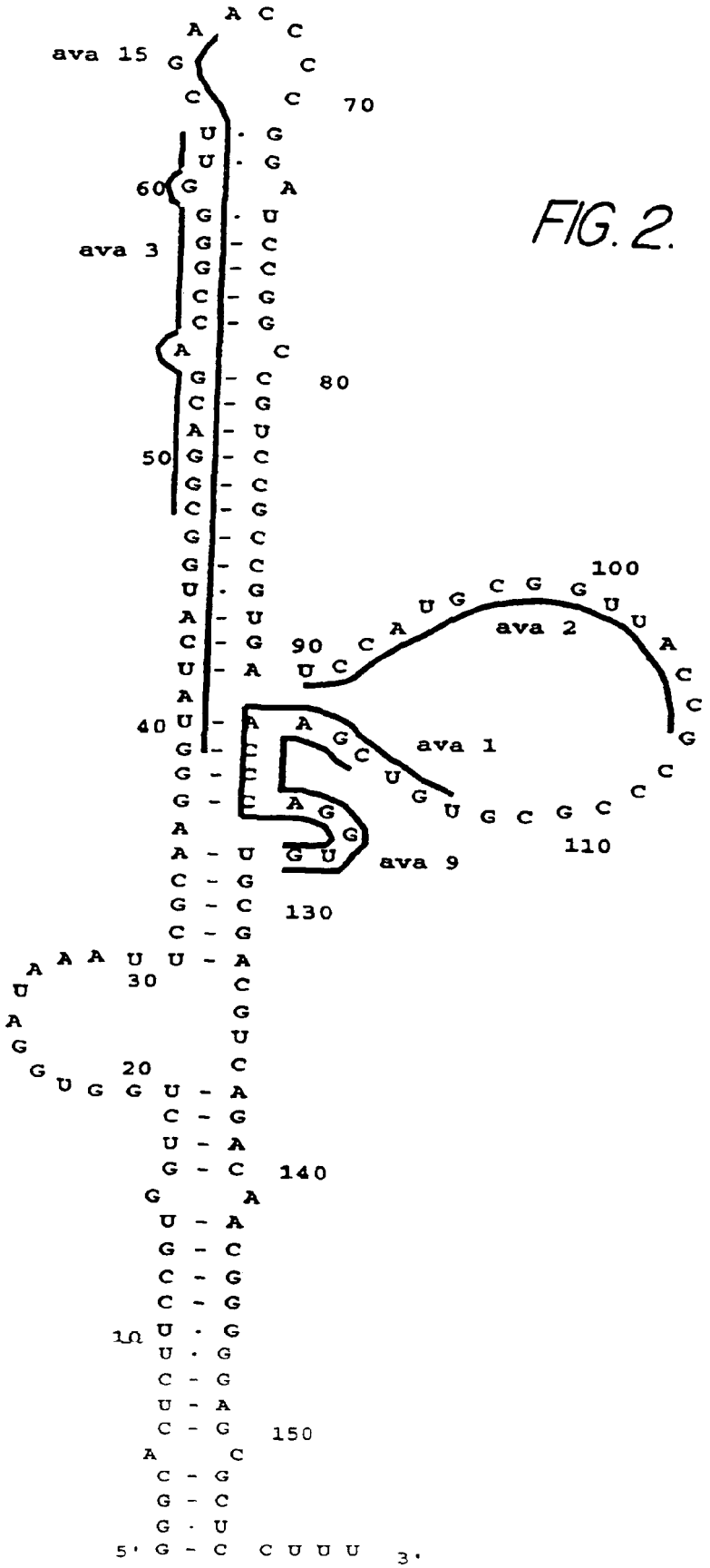

FIG. 2.

FIG. 3.

ava1		
5' CAC CTG GGT TCG ACA 3'	ODN	9/15 GC
3' GUG GAC CCA AGC UGU 5'	VA RNA	113-127
ava2		
5' CGG TAA CCG CAT GGA 3'	ODN	9/15 GC
3' GCC AUU GGC GUA CCU 5'	VA RNA	92-106
ava3		
5' AAC CCC GGT CGT CCG 3'	ODN	11/15 GC
3' UUG GGG CCA GCA GGC 5'	VA RNA	48-62
ava9		
5' CAC CTG GGT TCG 3'	ODN	8/12 GC
3' GUG GAC CCA AGC 5'	VA RNA	116-127
ava15		
5' TCG AAC CCC GGT CGT CCG CCA TGA TAC 3'	ODN	17/27 GC
3' AGC UUG GGG CCA GCA GGC GGU ACU AUG 5'	VA RNA	39-65

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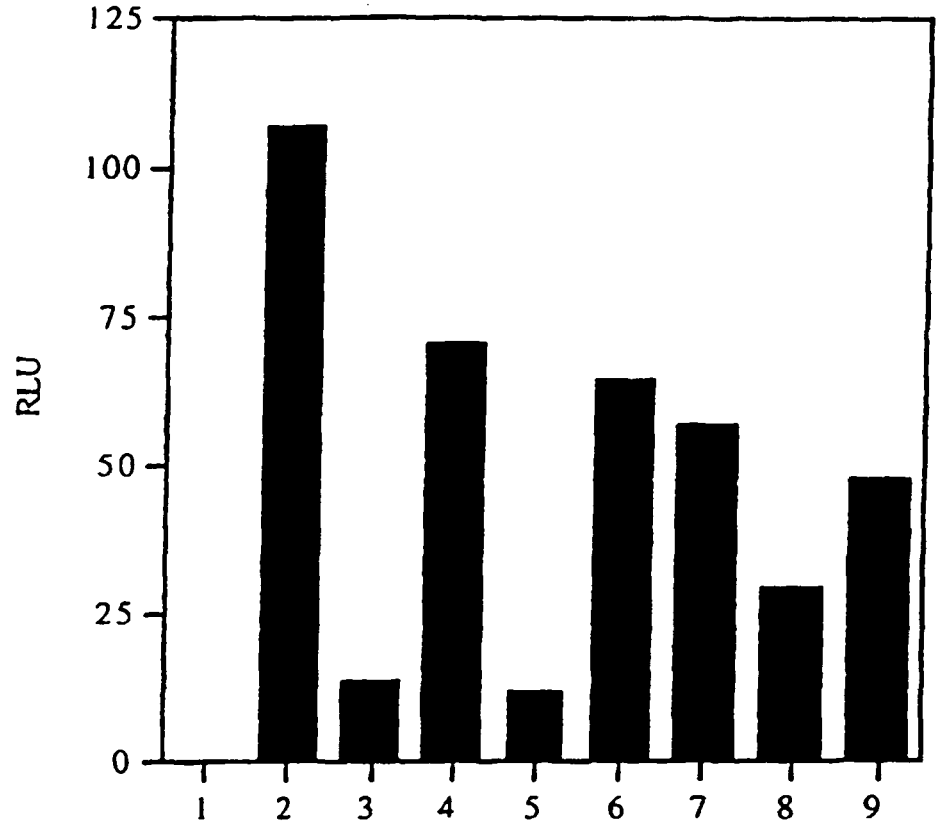
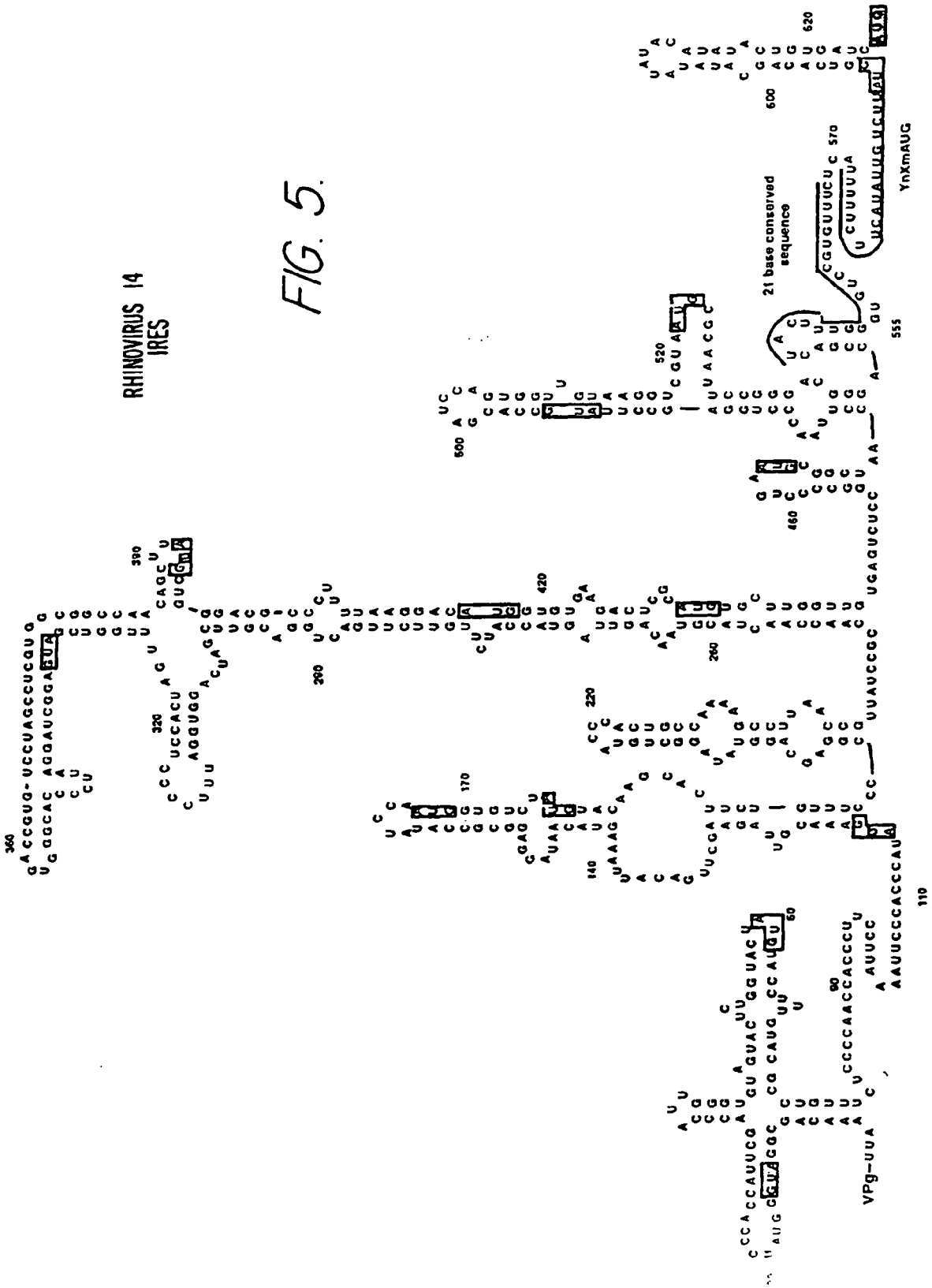


FIG. 4.

FIG. 5.


RHINOVIRUS 14
IRES
21 base conserved sequence
YnXmAUG
VPg-UUA

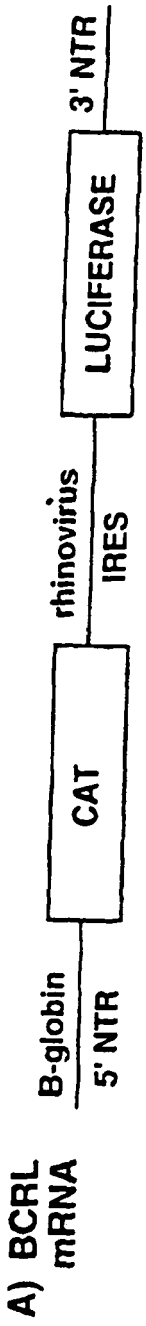

A) BCRL mRNA
B-globin 5' NTR
CAT
rhinovirus IRES
LUCIFERASE
3' NTR
B) BL mRNA
B-globin 5' NTR
LUCIFERASE
3' NTR

FIG. 6.

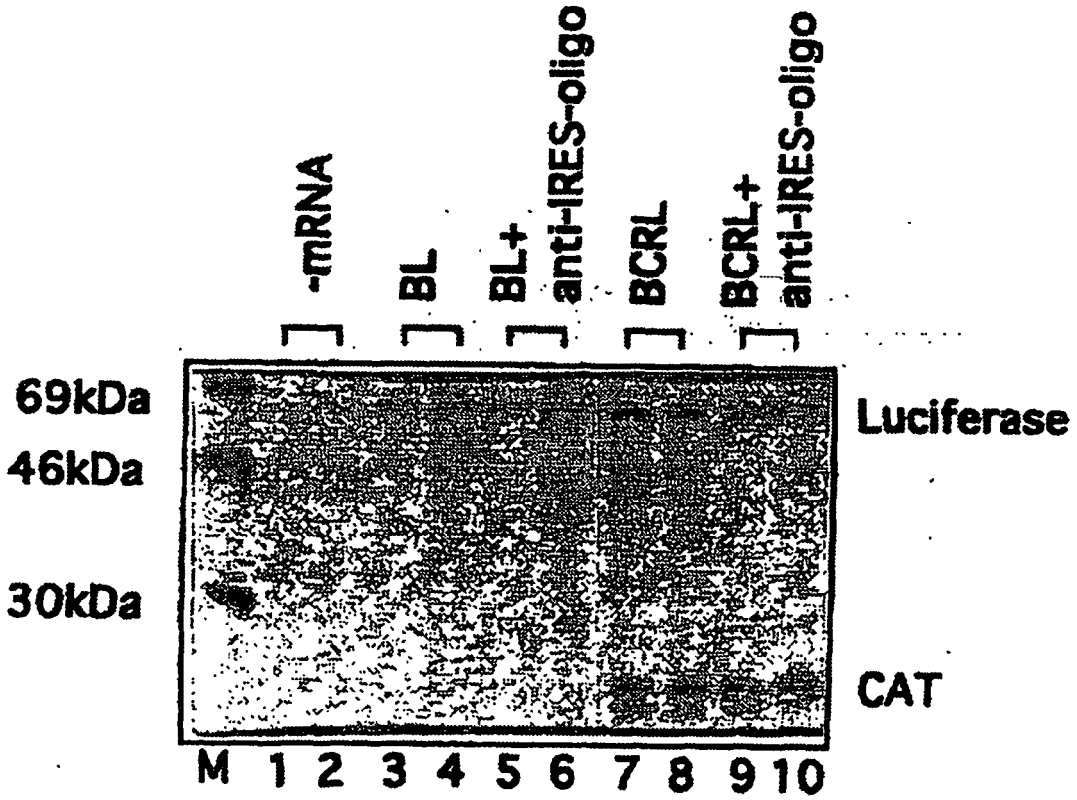

-mRNA
BL
BL+ anti-IRES-oligo
BCRL
BCRL+ anti-IRES-oligo
69kDa
46kDa
30kDa
Luciferase
CAT
M 1 2 3 4 5 6 7 8 9 10

FIG. 7.

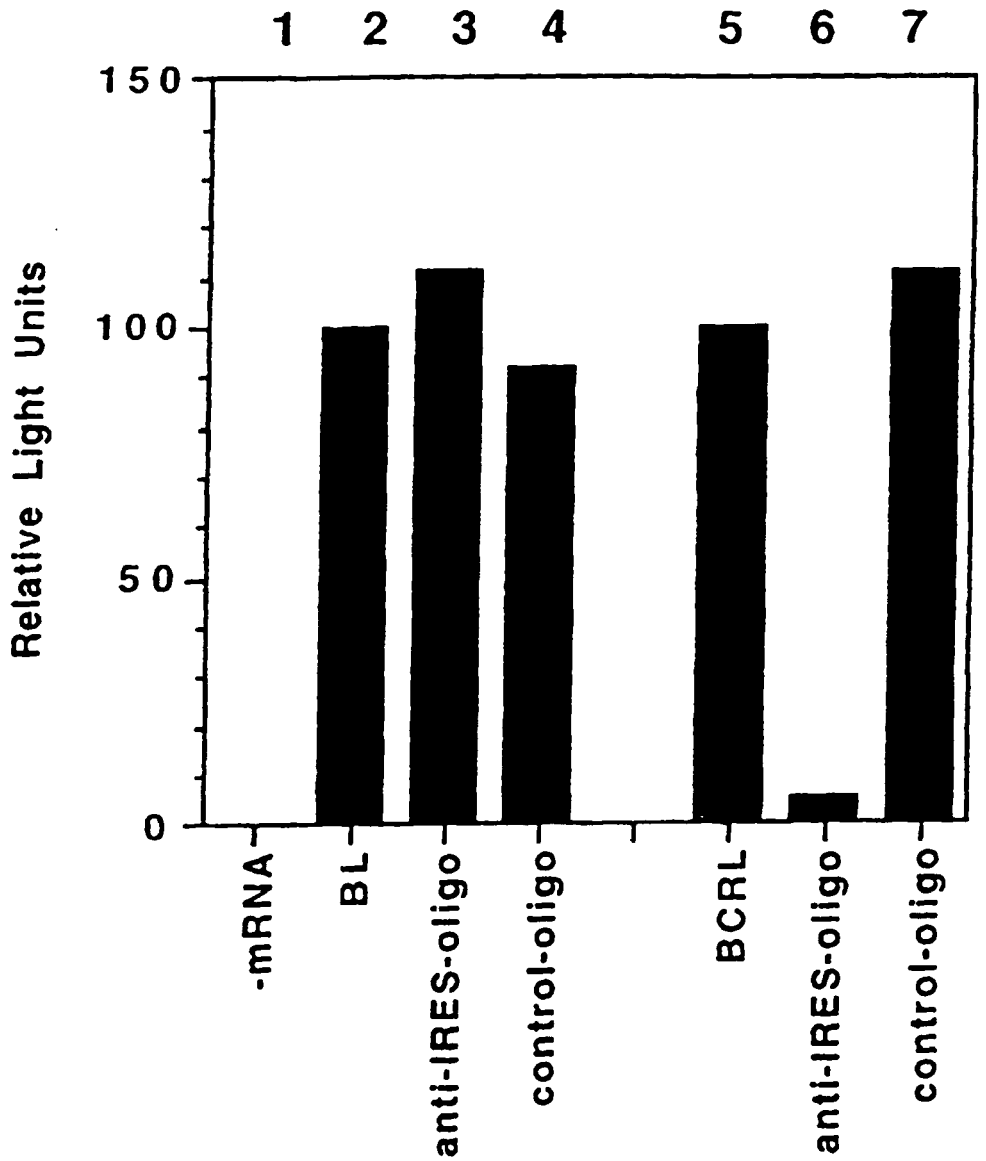

1
2
3
4
5
6
7
150
100
50
0
Relative Light Units
-mRNA
BL
anti-IRES-oligo
control-oligo
BCRL
anti-IRES-oligo
control-oligo

FIG. 8.

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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