

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
21 December 2006 (21.12.2006)

PCT

(10) International Publication Number
WO 2006/135447 A2

(51) International Patent Classification:

A61K 39/13 (2006.01)

(21) International Application Number:

PCT/US2005/040031

(22) International Filing Date:

4 November 2005 (04.11.2005)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

60/625,553 5 November 2004 (05.11.2004) US

(71) Applicant (for all designated States except US): **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin St., 12th Floor, Oakland, CA 94607-5200 (US).

(72) Inventors; and

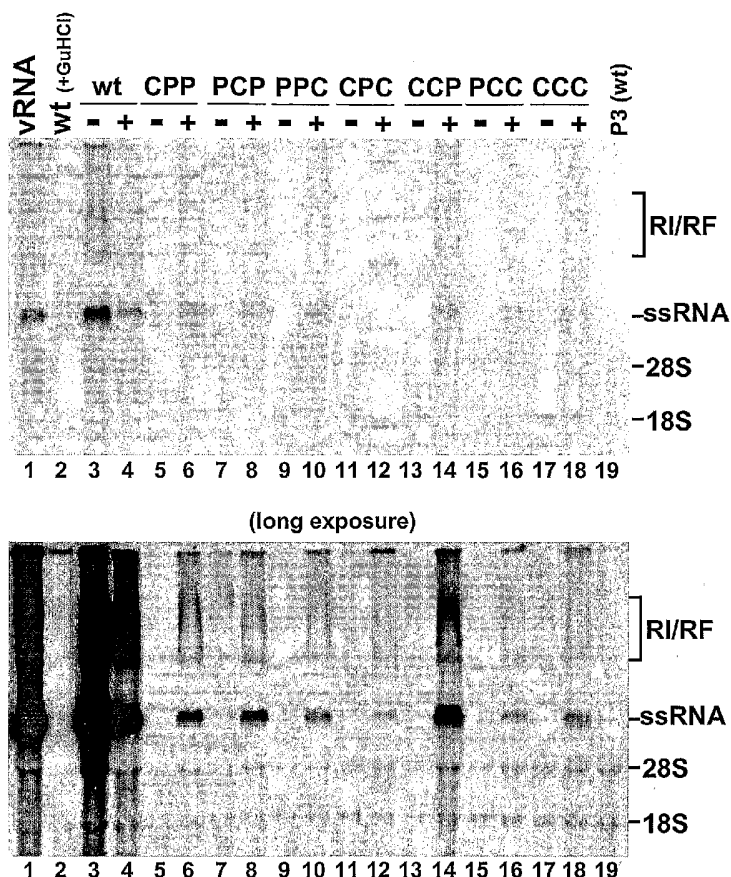
(75) Inventors/Applicants (for US only): **SEMLER, Bert L.** [US/US]; 904 Calle Venezia, San Clemente, CA 92672 (US). **CORNELL, Christopher** [US/US]; 1678 AvenidaOceano, Oceanside, CA 92056 (US). **BRUNNER, Jo Ellen** [US/US]; 18337 Mount Cherie Circle, Fountain Valley, US 92708 (US).(74) Agents: **FESSENMAIER, Martin** et al.; RUTAN & TUCKER, LLP, 611 Anton Blvd., Suite 1400, Costa Mesa, CA 92626 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

[Continued on next page]

(54) Title: VIRAL CHIMERA COMPOSITIONS AND METHODS



(57) Abstract: Chimeric nucleic acids are presented in which a portion of the polymerase gene or one picornavirus is replaced with the corresponding portion of another picornavirus. Remarkably, such constructs can produce positive and negative strand RNA *in vitro* when complemented by wild-type P3 polypeptide. Even more advantageously, such chimeric nucleic acids can be used to (a) determine mode of action of antiviral drugs when used with P3 mutants defective in IRES binding, protein processing, and/or 3D polymerase, (b) translate antiviral activity from one viral species to another species, and (c) investigate protein-protein and/or protein-RNA interactions.



European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:

- *without international search report and to be republished upon receipt of that report*

VIRAL CHIMERA COMPOSITIONS AND METHODS

This application claims priority to our copending U.S. provisional patent application with the serial number 60/625553, filed November 5, 2005, and which is incorporated by reference herein.

5 This invention was made with government support from the NIH using grant number AI 22693. The government may have certain rights in the invention.

Field of The Invention

The field of the invention is viral chimeric constructs of picornaviruses, and especially 3D chimeras of polio virus and coxsackie virus B3.

10 **Background of The Invention**

The intracellular replication cycles of picornaviruses typically involve multiple gene products of viral and host origins that alter the intracellular environment to make it suitable for rapid genome amplification and virion production. Most typically, the presence of these gene products results in shutdown of cellular protein synthesis and host transcription,
15 inhibition of Class I Major Histocompatibility Complex (MHC1) expression on the cell surface, alteration of nuclear import/export, membrane trafficking, and rearrangement and compartmentalization of host cell membranes. Remarkably, such complex functions are thought to be achieved by relatively few viral proteins and precursor polypeptides that interact with each other and host proteins to mediate distinct, highly specific functions in the viral life
20 cycle.

Poliovirus type 1 (PV1) is considered a prototypic member of the *Picornaviridae* and has a positive-sense single stranded RNA genome of ~7.4 kb, which contains a single open reading frame from which a 247 kDa viral polyprotein is translated by a cap-independent mechanism via internal ribosome entry. This viral polyprotein is processed by the two viral
25 proteinase functions within the polypeptide (2A and 3C, and in some cases 3CD precursor peptide) that are subsequently cleaved from the polyprotein. Still further proteolytic action releases then the remaining known polypeptide with distinct biochemical activity.

Figure 1A depicts a schematic illustration of the genome of PV1 and corresponding polypeptides. Here, regions encoding the capsid (structural) and non-capsid (non-structural)

proteins are at their respective position using nucleotide numbers. The corresponding viral polyprotein (247 kDa) is depicted with sites of proteolytic cleavage and precursor/mature cleavage products along with their estimated molecular weights. A diamond indicates the primary cleavage site liberating the P2-P3 polypeptide from the P1 polyprotein, the triangles indicate the Q-G dipeptide sites that are recognized and cleaved by 3C and 3CD proteinases, and the star indicates a cleavage site necessary for virion maturation (hydrolytic mechanism unknown). In early stages of viral protein processing, the 2A proteinase cleaves the structural precursor from the non-structural precursors, and further cleaves host factor eIF-4G, resulting in the shutdown of cap-dependent host protein synthesis. The majority of the other processing events within the viral polyprotein are then carried out by the 3C and/or 3CD proteinases.

Due to the limited number of genes present within a typical picornavirus genome, some precursor polypeptides have biochemical activities that differ greatly from those of their mature cleavage products. In addition, several precursor and mature cleavage products have been shown to be multifunctional, thereby increasing the effective coding capacity of the typical picornavirus genome. For example, the poliovirus 3CD polypeptide, a polyprotein containing the amino acid sequences of the 3C proteinase and the 3D RNA-dependent RNA polymerase, is an active proteinase that does not possess measurable RNA synthesis activity *in vitro*. In addition to proteolytic activity, the 3C and 3CD proteins contain determinants that mediate binding to viral RNA. 3CD has also been shown to interact with the 5'- and 3'-noncoding regions of the poliovirus genome, and is further thought to serve as a source of 3D polymerase within replication complexes utilized for RNA synthesis. Moreover, the 3CD polypeptide has also been shown to dramatically stimulate the process of VPg uridylylation, which may involve protein-protein and protein-RNA contacts between 3CD and the 3D polymerase, VPg (or the 3AB precursor polypeptide), and the cis-acting replication element within 2C (2C-cre).

In infected cells, poliovirus RNA synthesis may require the formation of replication complexes containing viral proteins that have been translated from the RNA around which they assemble. For example, coupling between translation and RNA replication was recently demonstrated by showing that viral genomes not actively translating are often not utilized as templates for RNA synthesis (even in the presence of fully replication-competent viral proteins translated from other genomes in the same cell). Furthermore, membranous vesicle formation (providing a platform on which RNA synthesis occurs) has been shown to be

coupled to viral RNA replication, translation, and encapsidation. Thus, it has been postulated that linking of protein synthesis to RNA replication may provide a mechanism by which the poliovirus (and likely other picornaviruses) selectively amplify RNA molecules that yield fully active viral proteins to thereby ensure production of progeny virions that contain viable
5 genomes.

While the above observations, hypotheses, and experiments provide at least some understanding in picornavirus replication, drug development and investigation into suitable drug targets has been hampered by the relative lack of knowledge of the rather complex viral replication mechanism. Among other things, the exact roles and interplay of the polypeptides
10 involved in positive and negative RNA strand synthesis are still not fully understood. Even where structural information is available, experimental evaluation of theoretical models from such information is often problematic due to various difficulties associated with *in vivo* and/or *in vitro* assays.

Thus, while numerous aspects and methods for picornaviruses are known in the art, all
15 or almost all of them suffer from one or more disadvantages. Therefore, there is still a need for model systems, and especially *in vitro* systems, that provide insight into viral replication requirements and drug action interfering with same.

Summary of the Invention

The present invention is directed to compositions and methods in which a chimeric
20 nucleic acid of a picornavirus is employed as a test system for drug discovery and analysis of mechanism of action of antiviral drugs. Most preferably, the chimeric nucleic acids are based on nucleic acids from a poliovirus and a coxsackie virus and are chimeric in the polymerase gene 3D.

In one aspect of the inventive subject matter, a test system has a plurality of chimeric
25 nucleic acids in which a first nucleic acid portion is from a first virus of a picornavirus family and in which a second nucleic acid portion is from a second virus of the picornavirus family, respectively, and wherein each of the second portions are sub-allelic portions of one or more viral genes of the second virus. Most preferably, the sub-allelic portions are selected such that a first and a second of the chimeric nucleic acids exhibit a distinct replication characteristic.
30 In certain preferred aspects, the chimeric nucleic acid is a double stranded DNA, and the test

system includes at least five, more typically at least six, and most typically at least seven nucleic acids. Additionally, contemplated systems may also include a nucleic acid encoding a wild-type P3 polypeptide, a P3 polypeptide that is defective in cloverleaf binding, protein processing, and/or polymerase function (for positive and/or negative strand RNA synthesis).

5 Particularly contemplated replication characteristics is characteristic for a negative and/or positive strand RNA synthesis and include one or more of an autonomous RNA replication, complementation by wild-type P3, complementation by P3 defective in cloverleaf binding, complementation by P3 defective in protein processing, and complementation by P3 with defective 3D polymerase. In such systems, the viral gene is the 3D gene, wherein the
10 sub-allelic portion covers at least one of three sections within the 3D gene. Where desirable, an instruction may be included in the system that instructs a user to test a pharmaceutically active compound in an *in vitro* experiment using at least one of the chimeric nucleic acids.

Therefore, in another aspect of the inventive subject matter, a method of providing a test system for test of an antiviral drug may include a step in which contemplated test systems
15 are provided. In another step, an instruction is provided to express at least one of the chimeric nucleic acids *in vitro* in the presence of an antiviral drug, and in yet another step, instructions are provided to optionally co-express at least one nucleic acid that encodes a polypeptide selected from the group consisting of a wild-type P3 polypeptide, a P3 polypeptide that is defective in cloverleaf binding, a P3 polypeptide that is defective in protein processing, and a
20 P3 polypeptide that has defective 3D polymerase function. In a still further step, instructions are provided to observe *in vitro* synthesis of at least one of a positive strand picornavirus RNA and a negative strand picornavirus RNA.

Most typically, contemplated methods will further provide instructions to correlate the synthesis of the positive strand or the negative strand picornavirus RNA with a mechanism of
25 antiviral action of the antiviral drug, and/or provide instructions to translate antiviral activity of the antiviral drug to another virus species based on the observation of *in vitro* synthesis of the positive strand or negative picornavirus RNA. In less preferred aspects, the step of providing instructions to express at least one of the chimeric nucleic acids *in vitro* in the presence of the antiviral drug is independently performed from the step of providing the test
30 system (*e.g.*, via a sales brochure). Additionally, it should be appreciated that the at least one nucleic acid that encodes the polypeptide selected from the group consisting of the wild-type

P3 polypeptide, the P3 polypeptide that is defective in cloverleaf binding, the P3 polypeptide that is defective in protein processing, and the P3 polypeptide that has the defective 3D polymerase function may be included in contemplated methods.

Thus, and viewed from a different perspective, a method of testing an antiviral drug
5 may include a step of expressing a chimeric nucleic acid *in vitro* in the presence of an antiviral drug, wherein a first nucleic acid portion of the chimeric nucleic acid is from a first virus of a picornavirus family, wherein a second nucleic acid portion of the chimeric nucleic acid is from a second virus of the picornavirus family, and wherein the second nucleic acid portion encodes at least a portion of a polymerase gene. In another step, synthesis of at least
10 one of a positive strand picornavirus RNA and a negative strand picornavirus RNA is measured. Most preferably, contemplated methods will typically further comprise a step of co-expressing at least one nucleic acid that encodes a polypeptide selected from the group consisting of a wild-type P3 polypeptide, a P3 polypeptide that is defective in cloverleaf binding, a P3 polypeptide that is defective in protein processing, and a P3 polypeptide that
15 has defective 3D polymerase function.

Various objects, features, aspects and advantages of the present invention will become more apparent from the following detailed description of preferred embodiments of the invention.

Brief Description of the Drawings

20 Figure 1A is a schematic illustration of the VPg-linked genome of poliovirus type 1 (PV1) and further illustrates the corresponding polyprotein and post-cleavage peptide components.

Figure 1B is a schematic illustration of exemplary 5' ribozyme PV1 cDNA (RzPV1) and PV1/CVB3 chimeras contemplated herein.

25 Figure 1C is a schematic illustration of exemplary pT7-5'-NCR-P3 transcripts utilized for *in vitro* RNA replication rescue experiments presented herein.

Figure 2A is an autoradiogram of rescued RNA replication in the presence of 5'-NCR-P3 (wt) co-translating RNA.

Figure 2B is an autoradiogram of [35S]methionine-labeled translations from the corresponding reactions shown in Figure 2A.

Figure 3A is an autoradiogram of RNA replication of wild type and chimeric transcripts with and without 5'-NCR-P3 (R13N) co-translating RNA.

5 Figure 3B is an autoradiogram of [35S]methionine-labeled translations from the corresponding reactions shown in Figure 3A.

Figure 4A is an autoradiogram of RNA replication of wild type and chimeric transcripts with and without 5'-NCR-P3 (C147A) co-translating RNA.

10 Figure 4B is an autoradiogram of [35S]methionine-labeled translations from the corresponding reactions shown in Figure 4A.

Figure 5A is an autoradiogram of RNA replication of wild type and chimeric transcripts with and without 5'-NCR-P3 (μ 61) co-translating RNA.

Figure 5B is an autoradiogram of [35S]methionine-labeled translations from the corresponding reactions shown in Figure 5A.

15 Figure 6 is a graph depicting results from *in vitro* RNA polymerase assays using wild-type and PV1/CVB3 transcripts to elongate nascent RNA chains from an oligo(U)-primed vRNA template.

Figure 7 is an exemplary summary table of RNA replication and complementation phenotypes of various PV1/CVB3 transcripts contemplated herein.

20 **Detailed Description**

The inventors unexpectedly discovered that defective RNA replication can be rescued *in vitro* by co-translating non-structural proteins from a transcript encoding a large precursor polyprotein (P3). In most contemplated aspects, replication is rescued where the replication is defective due to mutations and/or chimeric constructs in the viral 3D polymerase and/or
25 precursor polypeptide.

More specifically, and in one particularly preferred aspect, RNA replication is reduced or even entirely abolished by modification of the viral polymerase gene. Most preferably, the

modification in the viral polymerase gene is due to chimera formation, wherein the chimera is typically produced from genetic material of two distinct, but systematically related viruses. Furthermore, both viruses are preferably picornaviruses, wherein the first virus is a poliovirus (*e.g.*, PV1), and wherein the second virus is a coxsackie virus (*e.g.*, CVB3). Among other
5 unexpected results, the inventors discovered that the picornaviral 3D RNA polymerase gene is differentially responsive to trans rescue, depending on the particular lesion in the 3D gene. Such finding is particularly remarkable as non-differentiating complementations in trans have been performed for viral RNAs containing one or more mutations within non-structural gene products 2A, 2C, 3A, 3B (VPg), and 3C. Still further, complementation with contemplated
10 compositions and methods was in at least some cases even differentiating between positive and negative strand synthesis. Using genetically-modified forms of the complementing P3 polyprotein (*e.g.*, containing mutations within 3C or 3D sequences) strongly suggest the existence of different protein-protein and protein-RNA interactions required for positive-versus negative-strand RNA synthesis, which opens new avenues into rational drug design
15 and drug targeting for picornaviruses and other positive strand RNA viruses.

Contemplated Compositions

In one preferred aspect of the inventive subject matter, multiple distinct viral RNA genomes (*e.g.*, three, four, five, etc.) are contemplated in which one or more portions of the gene encoding the RNA polymerase of one viral species (recipient virus) is replaced with
20 corresponding portion(s) of the RNA polymerase gene of another viral species (donor virus).

For example, as depicted in exemplary **Figure 1B**, a chimeric viral RNA genome is derived from two picornaviruses, PV1 (poliovirus 1) and CVB3 (coxsackie virus B3). The schematic indicates the genetic organization of RzPV1, which allows *in vitro* transcription by bacteriophage T7 RNA polymerase and the production of a full-length PV1 transcript with a
25 precise 5' end. Below the cDNA schematic are the eight transcripts (wild type and chimeric) generated for *in vitro* translation and RNA replication studies presented in this work. Here, PV1/CVB3 RNA polymerase sequences were cloned into a full-length PV1 cDNA containing a 5'-hammerhead ribozyme sequence, which allows generation of transcripts from these constructs with precise 5' ends to thereby significantly increase the ability of PV1 transcripts
30 to synthesize positive-strand RNA. Most typically such constructs are linearized and *in vitro* transcribed using bacteriophage T7 RNA polymerase. In the case of the constructs of Figure

1A, seven distinct chimeric RNA genomes were obtained along with one wild-type RNA genome.

In alternative aspects of the inventive subject matter, it is contemplated that numerous viral species other than poliovirus and coxsackie virus may also be suitable as donors and/or recipients of a (preferably sub-allelic) portion of a genome. For example, especially suitable picornaviruses include various serotypes of human or bovine enteroviruses (*e.g.*, type A, B, C), human rhinoviruses, hepatoviruses, human parechoviruses, etc. Moreover, it should be appreciated that not only picornaviruses are contemplated, but that also other viruses, and especially RNA-viruses are appropriate for use herein. For example, (-)ssRNA viruses from various families, including paramyxoviridae, orthomyxoviridae, bunyaviridae, arenaviridae, rhabdoviridae, and filoviridae are suitable, and (+)ssRNA viruses from various families, including leviviridae, potviridae, hepeviridae, and caliciviridae. Further contemplated RNA viruses include those belonging to the family of coronaviridae.

In less preferred aspects, the donors and/or recipient of the (preferably sub-allelic) portion of the genome may also be non-RNA virus. In these instances, it is generally preferred that the recipient of the sub-allelic portion is an RNA virus, and most preferably a picornavirus. Thus, the choice of a suitable non-RNA virus is at least in part determined on residual polymerase activity in the chimeric construct, degree of nucleic acid identity in the chimeric portion between the donor and recipient, and/or the degree of amino acid identity or homology in the chimeric portion between the donor and recipient.

In further preferred aspects, it should be appreciated that the genetic material from the donor virus is within a single structural or non-structural gene of the virus, or where desired, also in a single 5'- and/or 3'-untranslated (non-coding) region. For example, preferred donor material is derived from a non-structural gene, and most preferably from a polymerase and/or peptidase in final processed form or precursor. Thus, suitable donor material is from the 3D, 3CD, or 3AB gene. In alternative aspects, however, more than one region may be used as a donor material, and all regions of a viral genome are considered suitable donor regions. Most typically the genetic material from the donor virus is a sub-allelic portion (less than the entire coding region) of a viral gene.

Therefore, it should be recognized that the exact boundaries and size of the donor material will vary considerably, and that the particular material will depend among other factors on the particular nature (*e.g.*, what is encoded) of the donor material. For example, sub-allelic boundaries of the donor material may be established by design where structural information from crystallography is available, or computed binding motifs, conformational analysis, hydrophilicity plots, or other physicochemical parameter are obtainable. On the other hand, sub-allelic (or other) boundaries of the donor material may also be established in a random manner to thereby generate a library of chimeric RNA genomes (or subgenomes). For example, random boundaries may be generated by PCR shuffling, *recA*-mediated processes, use of degenerated oligo(ribo)nucleotides, etc., and quasi-random borders may be employed by choice of suitable restriction sites.

In especially preferred aspects, the sub-allelic portion is of a gene under investigation, wherein that gene may have more than one function. For example, there are numerous known, suspected, and hypothesized functions for the 3CD polypeptide of picornaviruses and it can be assumed that various functions will be associated with one or more sub-domain (*e.g.*, with corresponding sub-allelic portion) within a gene. Therefore, sub-allelic gene exchange may modify such a function to a degree that is no more available for complementation by another protein, wherein that protein may be a wild-type protein or a mutated protein.

Consequently, it should be recognized that the length of the donor material will vary greatly and may be between a single nucleotide replacement (*e.g.*, transition or transversion) and several hundred (and less typically several thousand) bases, wherein the donor material may be inserted into the corresponding portions of the recipient viral genome in one or more positions and/or copies. Thus, it should be appreciated that the donor material is typically directly derived from a donor genome (*e.g.*, via restriction), or indirectly (*e.g.*, via PCR or synthetic DNA or RNA).

In at least some of contemplated chimeric constructs, one or more biological functions (*e.g.*, infectivity, capability of positive/negative strand RNA synthesis, etc.) will be lost or at least significantly reduced, and it is especially preferred that in such cases one or more complementing polypeptides (which may or may not be fully processed viral proteins) are provided that restore the biological functions to a least some degree. It should be noted that such complementation is especially advantageous where restoration is selective to the

particular type of chimeric construct. There are numerous complementation patterns in viruses, and especially RNA viruses/picornaviruses known in the art, and all of these patterns are deemed suitable for use herein. Furthermore, complementation may be performed in numerous manners, and especially preferred manners include co-translation of one or more
5 complementing peptides *in vivo* or *in vitro*, and addition of one or more complementing peptides *in vitro*.

However, in one particularly preferred aspect, at least partial functional loss of the 3D polymerase is restored by co-expression of P3 wild-type and/or P3 mutant polypeptide. Exemplary schematic illustrations of pT7-5'-NCR-P3 transcripts utilized for *in vitro* RNA
10 replication rescue experiments are depicted in **Figure 1C**. These constructs produce T7-derived, polyadenylated transcripts possessing the P3 (wild type or mutant) coding region translated by the PV1 internal ribosome entry site (IRES) and were previously described by Towner, et al. When present, the hatched region in each schematic indicates the location of the described point mutation.

15 Complementation was performed with P3 using 5'-NCR-P3 wild-type and various mutations (using constructs pT75'-NCR-P3 (wt), pT75'-NCR-P3 (C147A), pT75'-NCR-P3 (μ 61), pT75'-NCR-P3 (R13N) and pT75'-NCR-P3 (Y6N)) wherein the mutations were characterized by loss of specific functions (*e.g.*, defective in cloverleaf binding, protein processing, or polymerase function). Thus, it should further be recognized that contemplated
20 compositions not only provide a system in which the function of certain sequence portions of a viral gene can be probed using a complementation system, but that such systems also provide additional information of potential interaction of the viral gene portions (typically from a viral donor) with another viral gene or nucleic acid.

Consequently, the inventors contemplate a test system (*e.g.*, in form of a kit, or in
25 form of multiple available nucleic acids or portions thereof) that includes a plurality of chimeric nucleic acids in which a first nucleic acid portion is from a first virus (preferably of a picornavirus family) and in which a second nucleic acid portion is from a second virus (of the picornavirus family), respectively. In such systems, it is generally preferred that each of the second portions is a sub-allelic portion of one viral gene of the second virus, wherein the
30 sub-allelic portions are selected such that a first and a second of the chimeric nucleic acids exhibit a distinct replication characteristic (*e.g.*, capability of positive and/or negative strand

RNA synthesis with respect to autonomous RNA replication, complementation by wild-type P3, complementation by P3 defective in cloverleaf binding, complementation by P3 defective in protein processing, and/or complementation by P3 with defective 3D polymerase).

Among other preferred systems, especially preferred systems are addressed further below in the experimental section in which the viral gene is the 3D gene of picornaviruses (where the donor is CVB3 and the recipient is PV1), and wherein the 3D gene is divided in three sub-allelic portions (roughly corresponding to 1st, 2nd, and 3rd third). As pointed out above, it should be noted that more than one sub-allelic portion may be provided to the recipient, and in some cases, even an entire allele may be transferred from the donor to the recipient virus. Therefore, and depending on the number (and optionally position) of donor portions to the recipient virus, it should be recognized that the number of chimeric nucleic acids may vary considerably. However, for particularly contemplated test systems, it is generally preferred that the test system includes at least five, more typically six, and most typically seven distinct viral chimeras. Moreover, where the test system allows for at least partial functional complementation with another peptide (or other cellular component), it is generally preferred that such peptides (or nucleic acids encoding such peptides) are included in the test system.

Contemplated Uses

Based on the inventors' discovery that certain defects in the picornaviral 3D gene can be rescued in a distinct and characteristic manner using picornaviral P3 and certain mutations thereof, it is generally contemplated that the nucleic acids presented herein may be employed in an *in vivo*, and more preferably *in vitro* assay to investigate the mechanism of action of antiviral drugs and/or their target sites, to translate findings of one drug to in one virus or viral system to another virus (to thereby speed up the process of drug discovery), and/or to investigate protein-protein interactions and nucleic acid-protein interaction in the process of viral replication.

Thus, and viewed from a different perspective, contemplated systems can be used *in vitro* and/or *in vivo* to characterize one or more portions of a viral genome in terms of its function, and/or cooperation with other cellular components, including other viral processed proteins, viral precursor polypeptides (peptides that will be further processed in a cell, typically to yield at least one structurally and/or functionally different peptide, most typically

to yield two structurally and functionally distinct peptides), host cell peptides, and viral and/or host nucleic acids.

There are numerous suitable *in vitro* assay formats for use with the chimeric nucleic acids presented herein known in the art, and all of such systems are specifically contemplated herein. However, especially preferred systems are those described by Wimmer et al. in U.S. Pat. Nos. 5,674,729 and 6,264,940, both of which are incorporated by reference herein. Alternatively, cell-based *in vivo* assays may be employed and include those using human rhabdomyosarcoma cells, human larynx carcinoma cells, or Buffalo green monkey kidney cells following protocols well established in the art.

In still further contemplated uses, chimeric viral genomes or portions thereof (most preferably with sub-allelic donor portions) may be employed in combination with each other and/or in combination with P3 (and mutants thereof) to screen for binding partners of loss of binding interaction in the presence of a potential pharmaceutically active molecule. Among other suitable systems, particularly contemplated systems include yeast systems, and most typically yeast two-hybrid screening systems. Such systems will advantageously allow the identification of positive or negative interaction between the binding partners. Insights from such and other investigations can then be employed to synthesize drugs on a rational-design basis.

Therefore, the inventors contemplate a method of providing a test system for test of an antiviral drug (and/or for evaluation of protein-protein interaction or protein-nucleic acid interaction) in which contemplated test systems as described herein are provided to a user (or retailer). Most typically an instruction is also provided to express at least one of the chimeric nucleic acids *in vivo*, and more preferably *in vitro* in the presence of an antiviral drug, and to optionally co-express at least one nucleic acid that encodes a polypeptide selected from the group consisting of a wild-type P3 polypeptide, a P3 polypeptide that is defective in cloverleaf binding, a P3 polypeptide that is defective in protein processing, and a P3 polypeptide that has defective 3D polymerase function. A further instruction is then provided to observe the *in vivo* or *in vitro* synthesis of genomic viral nucleic acid (*e.g.*, positive or negative strand of a (picornavirus) RNA).

So obtained results (e.g., synthesis of positive or negative strand picornavirus RNA) may then be correlated with a mechanism of antiviral action of the antiviral drug. Once a mechanism of action of a drug is established, it should be appreciated that the drug or insight gained from the mode of action can then be translated to development of an antiviral drug in another viral species, genus, family, or even order. Of course, it should be recognized that such and other instructions (e.g., to express at least one of the chimeric nucleic acids *in vitro* in the presence of the antiviral drug) may be provided along with a kit (which may or may not include nucleic acids or peptides to complement a viral defect) or independently (e.g., in form of a flyer, publication, etc).

Therefore, viewed from a different perspective, a method of testing an antiviral drug may include a step in which a chimeric nucleic acid is expressed *in vitro* in the presence of an antiviral drug, wherein a first nucleic acid portion of the chimeric nucleic acid is from a first virus of a picornavirus family, and wherein a second nucleic acid portion of the chimeric nucleic acid is from a second virus of the picornavirus family, and wherein the second nucleic acid portion encodes at least a portion of a polymerase gene. In such methods, it is generally preferred to measure synthesis of the positive and/or negative strand picornavirus RNA. Where desirable, it should be recognized that in such methods at least one nucleic acid is co-expressed that encodes a polypeptide selected from the group consisting of a wild-type P3 polypeptide, a P3 polypeptide that is defective in cloverleaf binding, a P3 polypeptide that is defective in protein processing, and a P3 polypeptide that has defective 3D polymerase function.

Experiments and Results

Chimeric recombinant proteins with sub-allelic exchanges of polymerase sequences between poliovirus type 1 (PV1) and coxsackievirus B3 (CVB3) were utilized to understand the RNA binding and protein processing activities of 3CD and/or 3D. Among other things, the results below demonstrate the effects of these 3D mutations on viral RNA replication *in vitro*, providing not only an indication of polymerase activity (with respect to both positive and negative strand synthesis), but also for the ability of the 3D polymerase and polymerase precursor polypeptides to assemble into functional complexes required for RNA synthesis. Three of the seven chimeric constructs showed detectable levels of RNA synthesis *in vitro*. However, it should be noted that not all of the chimeric constructs were capable of both

negative- and positive-strand RNA synthesis. Although *in vitro* 3D elongation assays confirmed that several of the polymerases themselves are somewhat defective at the level of RNA chain elongation, complementation experiments utilizing wild type P3 precursor protein provided in trans demonstrated that all of the chimeric transcripts were responsive to a rescue of both negative- and positive-strand RNA synthesis. Moreover, it should be appreciated that contemplated systems (*e.g.*, utilizing mutated forms of P3) also revealed differences in macromolecular interactions (protein-protein and protein-RNA) between proteins in cis and those in trans.

Materials and Methods

Plasmids and cloning: Chimeric PV1/CVB3 polymerase sequences were originally cloned as luciferase replicon constructs based on a modified version of a previously published plasmid, pRib(+)*RLuc*. pRib(+)*RLuc* was first digested with *Bgl*II and *Mlu*I in the presence of alkaline phosphatase (Promega). The ~8.0 kb vector fragment was gel purified and incubated with the ~1.8 kb fragment from an equivalent digest of pT7PV1(*Mlu*I) in the presence of T4 DNA ligase to generate pRib(+)*RLucM*. This construct was then re-digested with *Bgl*II and *Mlu*I in the presence of alkaline phosphatase (Promega) and the ~8.0 kb vector fragment was gel purified. The vector fragment was incubated in a four fragment ligation with the *Bgl*II-*Bsa*I fragment from pT7PV1(*Mlu*I), the *Bsa*I-*Mly*I fragment from pET15b-3CD (PCP), pET15b-3CD (PCC), pET15b-3CD (CCP), or pET15b-3CD (CCC) and the *Mly*I-*Mlu*I fragment from pRib(+)*RLucM* to generate the PCP, PCC, CCP, and CCC versions of pRib(+)*RLucM*.

To generate the CPP, PPC, and CPC versions of this construct, four fragment ligations were assembled with the same vector fragments from pRib(+)*RLucM* and pT7PV1(*Mlu*I), the *Bsa*I-*Xcm*I fragment from pET15b-3CD (CPP), pET15b-3CD (PPC), or pET15b-3CD (CPC) and the *Xcm*I-*Mlu*I fragment from pRib(+)*RLucM*. Once luciferase replicon constructs harboring the PV1/CVB3 chimeric sequences were generated, full length constructs were made by cloning the capsid coding sequences in place of luciferase. To accomplish this, the chimeric pRib(+)*RLucM* plasmids were digested with *Age*I and *Bgl*II and the ~5.5 kb vector fragment was then ligated to the corresponding *Age*I-*Bgl*II fragment from pT7PV1(*Mlu*I). All plasmids generated contain a 5' hammerhead ribozyme sequence immediately upstream of the PV1 sequence, and either wild type [RzPV1(wt)] or chimeric 3D polymerase sequences

[e.g., RzPV1 (CPP)]. All constructs were verified by restriction enzyme digests and nucleotide sequencing (Biotech Diagnostic; Laguna Niguel, CA).

P3 containing plasmids and mutant forms of P3 in plasmids (pT75'-NCR-P3 (wt), pT75'-NCR-P3 (C147A), pT75'-NCR-P3 (μ 61), pT75'-NCR-P3 (R13N) and pT75'-NCR-P3 (Y6N)) have been previously described.

In vitro synthesis of chimeric and mutated transcripts: To generate templates for *in vitro* transcriptions, full length PV1/CVB3 chimeric constructs (RzPV1) were linearized with MluI whereas pT75'-NCR-P3 plasmids were linearized with AatII followed by end-fill repair with the Klenow fragment of DNA polymerase I. Each linearized template was phenol/chloroform extracted, ethanol precipitated, washed with 70% ethanol, and resuspended in diethylpyrocarbonate-treated water. *In vitro* transcription reactions were carried out using bacteriophage T7 RNA polymerase at 37°C in a total volume of 20 μ l. Following incubation for one hour, reactions were treated with 2 units of DNase and incubated for an additional 15 minutes at 37°C. Reactions were quenched with the addition of 375 μ l of SDS stop buffer [0.5% SDS, 100 mM NaCl, 10 mM TRIS-HCl (pH 7.5), 1 mM EDTA] and 100 μ g of pre-digested proteinase K. This mixture was incubated at 37°C for an additional 30 minutes, phenol/chloroform extracted, and ethanol precipitated. RNA pellets were washed with 70% ethanol, dried, and resuspended in diethylpyrocarbonate-treated water. All transcripts were quantitated on an ethidium bromide stained agarose gel using transcript RNA of the same length and known quantity as a standard.

In vitro translation and RNA replication reactions: For translation/RNA replication experiments, 50 μ l reactions were assembled containing 65% (vol/vol) HeLa S10 cytoplasmic extract, 1.0 μ g of wild type RzPV1 or chimeric RzPV1 RNA, 10% (vol/vol) of 10 $\times$ replication mix [10 mM ATP, 2.5 mM GTP, 2.5 mM UTP, 600 mM potassium acetate, 300 mM creatine phosphate (Boehringer Mannheim), 4 mg of creatine kinase (Boehringer Mannheim) per ml, 155 mM HEPES-KOH (pH 7.4)], and 2 mM guanidine hydrochloride. Co-translation reactions contained 1.0 μ g of full length RzPV1 RNA plus an equimolar amount (400 ng) of 5'-NCR-P3 RNA. For translation analysis, 10 μ l of this reaction was added to 10 μ Ci of [35S]methionine (>1,000 Ci/mmol; Amersham Pharmacia Biotech). The remaining 40 μ l was used for RNA replication analysis. Both reactions were incubated at 30°C for 5 hours, at which time 10 μ l of 2X Laemmli sample buffer was added to each

translation reaction. The samples were boiled and resolved on a 12.5% polyacrylamide gel containing sodium dodecyl sulfate, fluorographed, and subjected to autoradiography on X-MR film (Kodak). The 40 µl RNA replication reactions were subjected to centrifugation for 20 minutes at 15,000 x g at 4°C, and the supernatants were removed. Pellets containing replication complexes were resuspended in 9 µl of fresh HeLa S10 cytoplasmic extract, 1.3 µl of 10X replication mix, and 2.5 µl (25 µCi) of [α -³²P]CTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) and incubated for 2 hours at 34°C. Following incubation, total RNA in each reaction was isolated by RNeasy spin column purification (Qiagen), subjected to a final ammonium acetate precipitation, washed with 70% ethanol, resuspended in 10 µl diethylpyrocarbonate-treated water/RNA loading buffer (Ambion) and subjected to gel electrophoresis on a native 1% agarose TBE gel containing ethidium bromide. The levels of 18S and 28S ribosomal RNA present in each lane were used to confirm equal loading of samples before the gel was dried and subjected to PhosphorImager analysis on a Personal Molecular Imager FX (BioRad).

3D polymerase *in vitro* elongation assays: For *in vitro* 3Dpol elongation assays, the inventors first carried out *in vitro* translations by slight modifications of previously published procedures. Translation reactions (25 ml) were assembled containing 60% (vol/vol) HeLa S10 cytoplasmic extract, 5% (vol/vol) HeLa ribosomal salt wash, 12 U RNasin, 10% (vol/vol) 10X replication mix, and 0.7 mg of wild type RzPV1 or chimeric RzPV1 RNA. Following incubation at 32°C for 6 hours, 3 ml of each translation was used in a subsequent elongation reaction consisting of 10% (vol/vol) 10X elongation buffer [50 mM HEPES-KOH (pH 8.0), 10 mM dithiothreitol, 3 mM MgCl₂, 300 mM each ATP, GTP, and UTP], 2 mg PV1 virion RNA, 30 ng oligo(U), and 10 mCi [α -³²P]CTP adjusted to 5 mM with unlabeled CTP in a final volume of 20 ml. The reactions were incubated at 30°C, and at 0, 15, 30, and 60 min., 4 ml was spotted onto a DE 81 paper circle (Whatman). The circles were washed three times in 5% dibasic sodium phosphate buffer, once in distilled water, rinsed in methanol, and air-dried. Incorporation of labeled nucleotides was measured by liquid scintillation counting, and the counts above background were calculated for each sample.

Results

Infectivity analysis of chimeric PV1/CVB3 transcripts: Chimeric PV1/CVB3 RNA polymerase sequences were cloned into a full-length PV1 cDNA containing a 5' hammerhead

ribozyme sequence (see Figure 1B). Inclusion of the 5' hammerhead ribozyme sequence allows the generation of transcripts from these constructs with precise 5' ends to significantly increase the ability of PV1 transcripts to synthesize positive-strand RNA. Linearization of these plasmids with Mlu I followed by *in vitro* transcription with bacteriophage T7 RNA polymerase generated eight different RNA transcripts (see Figure 1B). The inventors first examined the ability of each transcript RNA to yield infectious virus in cell culture at 33°C or 37°C by transfection of HeLa cell monolayers. Whereas the wild type transcript resulted in complete cytopathic effects (CPE) at approximately 18 hours post-transfection, none of the chimeric transcripts yielded CPE or detectable virus even after harvesting and freeze-thawing the monolayers as late as ~4 days post-transfection (data not shown).

Translation and RNA replication of PV1/CVB3 transcripts with and without P3 (wt) RNA: In earlier experiments (data not shown), the inventors analyzed the ability of various chimeric transcripts to replicate autonomously in an *in vitro* translation and RNA replication assay. **Figure 2A** depicts rescue of RNA replication by all chimeras in the presence of 5'-NCR-P3 (wt) co-translating RNA. Here, [α -32P] -labeled RNA replication reactions were programmed with 1.0 μ g full length wild type or chimeric RzPV1 transcript RNA as described below. The plus signs indicate reactions that were programmed with an equimolar (~0.4 μ g) amount of 5'-NCR-P3 (wt) RNA. Figure 2A is an autoradiograph of a non-denaturing agarose gel. Lane 1 shows a positive control utilizing RNA purified from poliovirus virions (vRNA). Lane 2 is a negative control in which the wild type RzPV1 replication reaction was supplemented with 2 mM guanidine hydrochloride (GuHCl). The identity of the full-length RzPV1 transcript RNA utilized in each reaction is indicated above each lane. Lane 19 is a control reaction utilizing ~0.4 μ g of 5'-NCR-P3 (wt) transcript alone. The mobilities (visualized by ethidium bromide staining) of single stranded virion RNA (ssRNA) and the 28S and 18S ribosomal RNAs are indicated to the right of the panel. The RNA species migrating slightly slower than the ssRNA species in some lanes (e.g., lanes 1 and 3) in this and other subsequent figures most likely represents a variant isoform of full length viral RNA. Also shown is the predicted mobility of the replicative intermediate/replicative form (RI/RF), corresponding, in part, to negative-strand RNA synthesis. The second autoradiograph shown below is a longer exposure of the same gel.

Figure 2A shows that the CPP chimera synthesizes only very low levels of negative-strand RNA (labeled "RI/RF") in the absence of positive-strand (labeled "ssRNA") RNA

synthesis (Figure 2A, lane 5). Two other chimeras (PCP and CCP) showed both negative- and positive-strand RNA synthesis, albeit at much lower levels when compared to replication of wild type PV1 RNA (Figure 2A, compare lanes 7 and 13 to lane 3). A positive-strand RNA signal for CCP becomes evident only with an extremely long exposure of the gel shown in Figure 2A (data not shown) and is apparent in results presented below (*e.g.*, Figure 4A, lane 13).

The remaining four chimeras (PPC, CPC, PCC and CCC) showed no detectable levels of RNA synthesis (Figure 2A, lanes 9, 11, 15 and 17). These results demonstrate differences in the ability of each chimeric 3D and/or 3CD polypeptide to effectively carry out one or more functions necessary for RNA replication *in vitro*. Furthermore, since transfected PCP and CCP transcripts do not yield virus in HeLa cell monolayers, there is likely an inability for these chimeras to generate sufficient threshold amounts of positive-strand RNA in cells, or perhaps a packaging or assembly defect preventing the production of new virions.

The inventors also carried out experiments with wild type and chimeric transcripts lacking a precise 5' end generated from cDNAs that do not contain a 5' ribozyme sequence. The 5' ends of these transcripts contain two guanosine nucleotides required by the T7 RNA polymerase and previously shown to reduce the levels of positive-strand RNA synthesis. By using this additional set of constructs in similar experiments, the inventors were able to confirm that the signal labeled "RI/RF" in Figure 2A corresponds, at least in part, to the synthesis of negative-strands (data not shown), verifying the RNA synthesis phenotypes described above.

The range of RNA replication phenotypes of these chimeras raises several questions about the macromolecular interactions involving the 3D polymerase and/or 3CD polypeptide required for positive- versus negative-strand RNA synthesis. Therefore, the inventors carried out additional translation-RNA replication reactions in the presence of an IRES-driven RNA expressing the entire wild type P3 precursor polypeptide [5'NCR-P3 (wt); see Figure 1C]. Based in part on earlier results (data not shown) involving *in vitro* complementation of non-structural gene functions, the inventors predicted that the P3 (wt) precursor polypeptide would be utilized in trans as a source of wild type 3D polymerase or precursor (*i.e.*, 3CD) proteins and could complement deficient chimeric 3D polymerases that are inactive in RNA recognition and elongation, as well as those chimeric 3CD polypeptides defective in RNA

binding or protein processing. Importantly, this approach should yield insights into the ability of wild type proteins provided in trans to interact with and form functional replication complexes with the viral RNA, P2 region polypeptides, and chimeric proteins provided in cis.

Results from complementation studies using the 5'NCR-P3 (wt) RNA are shown in Figures 2A and 2B. Figure 2A shows that the defective RNA replication phenotypes of each chimeric transcript can be complemented in trans by the co-expression of a wild type P3 precursor protein (lanes 3-18, compare odd to even numbered lanes), albeit with different efficiencies (for example, compare PCC to CCC; lanes 16 and 18). The presence of the wild type P3 RNA slightly reduced the ability of wild type RzPV1 transcript to synthesize RNA (compare lanes 3 and 4), perhaps reflecting a competition of proteins binding to P3 versus full-length transcripts. An increase in the detectable levels of both RI/RF and ssRNA was observed in each chimera in the presence of the rescue RNA encoding P3(wt), suggesting that wild type proteins provided in trans are either capable of recognizing the chimeric RNA on their own (in the absence of chimeric proteins), or are capable of forming functional hetero-complexes with chimeric proteins capable of initiating RNA synthesis. By itself, the CPP chimera was unable to synthesize positive-strand RNA, but this construct displayed positive-strand RNA synthesis in the presence of the wt P3 precursor protein (compare lanes 5 and 6). Rescue occurred to the same level as with PCP, a chimera capable of low levels of positive-strand RNA synthesis in the absence of wt P3 (compare lane 6 to 8), indicating these two chimeras are able to participate in equally productive interactions with wild type proteins provided in trans. Results with chimeras not as responsive to P3-mediated rescue (i.e., CPC and PCC; lanes 12 and 16) suggest a reduced ability of those chimeric proteins to form functional interactions with their wild type binding partners or to act as dominant-negative inhibitors by forming some non-functional protein-protein or protein-RNA complexes. Lane 19 of shows the inability of the P3 (wt) RNA to replicate on its own, demonstrating that the RNA synthesis observed in each reaction represents the amplification of only full length RzPV1 transcripts.

The translation results for the P3 (wt) rescue experiment are shown in **Figure 2B**. Here, [35S]methionine-labeled translations from the corresponding reactions shown in Figure 2A. Full length wild type or chimeric RzPV1 transcripts were incubated in the presence and absence of 5'-NCR-P3 (wt) rescue RNA. The identities of the precursor and mature cleavage products generated during translation and processing in vitro are indicated on the left side of

the panel. Note the different mobilities of the chimeric 3CD and 3D polypeptides in each lane due to the substitution of PV1 amino acids with those from CVB3. Lane 19 shows the products from the reaction programmed with 5'-NCR-P3 (wt) RNA alone (full length P3, 3BCD and 3CD precursor polypeptides, respectively). A longer exposure of this gel reveals the presence of mature 3A protein (data not shown).

These results verify that the differences observed in RNA replication among the chimeric transcripts in Figure 2A are not due to inherent differences in translation levels (lanes 5-18). Furthermore, the presence of the P3 co-translating RNA does not inhibit the synthesis of gene products from the full length RNAs (lanes 3-18, compare odd to even numbered lanes). Lane 19 of Figure 2B shows the proteins generated from a translation incubated with 5'-NCR-P3 (wt) transcript alone and demonstrates the production of full length P3 precursor protein and two major processing intermediates, 3BCD and 3CD (the overall translation level and methionine content of the 3A protein prevent it from being seen here). The inventors therefore conclude that the co-translated P3 precursor protein provides full length P3 protein as well as several processing intermediates to each reaction.

The inventors have previously investigated the biochemical properties of recombinant 3CD polypeptides harboring each of the chimeric polymerase sequences (data not shown). The chimeras CPP, PPC, CPC, PCC, and CCC displayed a P1 (capsid) processing deficiency in this assay, indicated by the level of uncleaved VP0-VP3 precursor protein compared to mature cleavage products VP0 and VP3 observed in the translation reactions (Figure 2B, lanes 5, 9, 11, 15 and 17). Furthermore, there are no other detectable processing defects that could prohibit RNA replication. In the samples that contained the 5'-NCR-P3 (wt) RNA, the wild type 3CD polypeptide provided in trans is capable of processing the uncleaved VP0-VP3 polypeptide to completion (compare levels of VP0-VP3 with and without P3 RNA for CPP, PPC, CPC, PCC and CCC). This provides direct evidence for the ability of proteins encoded by the full length transcripts to interact with those produced from the sub-genomic P3 RNA (i.e., 3CD).

Translation and RNA replication of PV1/CVB3 transcripts in the presence of P3 (R13N) RNA: The inventors then determined if the efficiency of RNA replication rescue achieved by co-translation of a wild type P3 RNA could be altered by the use of a mutant form of P3. 5'-NCR-P3 (R13N) (see Figure 1C) encodes a P3 protein harboring a previously

described point mutation in 3C that abrogates the ability of 3C to bind the PV1 RNA cloverleaf (stem-loop I) at the very 5' end of the positive-strand without affecting the ability of 3CD to process polyprotein. Therefore, this version of P3 can serve as a source of wild type 3D RNA polymerase and produce a 3CD precursor polypeptide able to process polyprotein but unable to bind RNA. The results of the RNA replication reactions in the presence and absence of P3 (R13N) are shown in **Figure 3A**. Here, RNA replication was carried out as described for Figure 2A, utilizing a 5'-NCR-P3 (R13N) transcript in each co-translation reaction (indicated with a plus sign). The autoradiogram shown below is a longer exposure of the same gel.

Consistent with the results using the wild type P3 construct, both CPP and PCP rescued to similar levels of RNA synthesis with the P3 (R13N) RNA (compare lane 5 to 6 and 7 to 8). The block to positive-strand RNA synthesis by the CPP chimera is relieved in the presence of P3 (R13N) (compare lanes 5 and 6). Chimera CCP also rescued to a similar level to that observed with the wt P3 protein (compare lanes 13 and 14 in Figure 2A and 3A). Therefore, rescue of these chimeras is not related to the ability of 3CD provided in trans to bind the cloverleaf structure near the 5' end of the viral RNA. The accessibility of 3CD (wt or R13N) to sites of negative- and positive-strand RNA synthesis initiation may be mediated completely by protein-protein contacts with chimeric 3CD molecules bound to the RNA in cis. In contrast, PPC and CPC show a dramatically reduced ability to be rescued by P3 (R13N) (compare lane 9 to 10 and 11 to 12). This could reflect a deficiency in protein-protein contacts between 3CD (R13N) and 3CD (PPC), as well as 3CD (R13N) and 3CD (CPC), resulting in an inability of 3CD (R13N) to be recruited to initiation complexes. Furthermore, PPC and CPC could encode polymerases defective in RNA synthesis initiation or elongation which could oligomerize with wild type 3D provided in trans, resulting in higher-order RNA polymerase structures reduced in overall activity. Finally, protein-protein interactions between PCC and CCC chimeric proteins may not be sufficient for even low levels of trans protein recruitment, resulting in a complete lack of RNA synthesis in the presence of P3 (R13N) (lanes 15-18). As expected, P3 (R13N) is incapable of autonomous RNA replication (lane 19).

As can be taken from **Figure 3B**, the translation and processing results for this rescue experiment appear to mirror those observed in the P3 (wt) rescue assay (compare Figure 2B to 3B). Here, [35S]methionine-labeled translations from the corresponding reactions shown in

Figure 3A, carried out as in Figure 2B with and without 5'-NCR-P3 (R13N). As expected, 3CD (R13N) is able to trans process the VP0-VP3 precursor polypeptide to completion (Figure 3B, lanes 6, 10, 12, 16 and 18). Another 5'-NCR-P3 mutant construct containing a different lesion in 3C [5'-NCR-P3 (Y6N)] also known to abrogate RNA binding was utilized in similar translation and RNA replication experiments. Complementation with the P3 (Y6N) construct yielded results (data not shown) very similar to the data shown in Figure 2.

Translation and RNA replication of PV1/CVB3 transcripts in the presence of P3 (C147A) RNA: To directly test whether a 3CD polypeptide provided in trans requires proteolytic activity to rescue chimeric RNA replication, the inventors used a construct harboring a mutation in 3C [5'-NCR-P3 (C147A)] (see Figure 1C) that destroys the proteolytic cleavage activity of the 3CD molecule. Processing of this version of P3 would be entirely dependent on the proteolytic activity of each chimeric 3CD polypeptide, since cleavage of P3 (C147A) cannot occur in cis. Following trans processing by the chimeric 3CD polypeptide, P3 (C147A) yields a wild type 3D polymerase and a proteolytically inactive 3CD molecule that retains its ability to bind cloverleaf RNA. Results from translation and replication reactions in the presence and absence of P3 (C147A) are shown in the figures below. As can be seen in **Figure 4A**, the presence of a co-translating P3 (C147A) transcript reduced the efficiency of wild type RNA synthesis (compare lanes 3 and 4). Here, RNA replication carried out as described for Figure 2A, utilizing a 5'-NCR-P3 (C147A) transcript in each co-translation reaction (indicated with a plus sign). The autoradiogram shown below is a longer exposure of the same gel.

Approximately equal levels of rescue were observed with the CPP and CCP chimeras in the presence of the P3 (C147A) RNA (compare lane 5 to 6 and lane 13 to 14). This suggests that the ability of P3 (C147A) proteins to interact with these two sets of chimeric polypeptides is similar, resulting in the recruitment of chimeric 3CD molecules to active sites of negative- and positive-strand RNA synthesis. Processing of 3CD (C147A) by the chimeric 3CD polypeptides must occur, providing a source of active, wild type polymerase to sites of RNA synthesis initiation. The PCP chimera did not rescue to the same level as CPP and CCP (compare lane 8 to lanes 6 and 14), suggesting that protein-protein interactions required to recruit P3 (C147A) polypeptides to sites of replication are not efficient with the PCP chimera. Comparing this result to the rescue of PCP using P3 (R13N) shown in Figure 3A, the inventors hypothesize that the ability of the 3CD molecule provided in trans to process itself

could be a factor in determining the susceptibility of this chimera to complementation. This conclusion can be extended to the results with the PPC and CPC chimeras, which also do not rescue to the same levels with P3 (C147A) compared to P3 (R13N) (compare lanes 9-12 in Figure 3A and 4A). Alternatively, the results with the PCP, PPC, and CPC chimeras could suggest that a 3CD molecule capable of binding RNA in the context of a replication complex but unable to process itself to yield 3D polymerase inhibits the overall function of that complex. Finally, P3 (C147A) does not rescue RNA replication of the PCC and CCC chimeras to levels detectable by this assay (lanes 15-18). Since this result is independent of the ability of P3 proteins provided in trans to bind RNA (compare to Figure 3A, lanes 15-18), the inventors again conclude that the PCC and CCC chimeric proteins do not participate in productive protein-protein interactions with trans binding partners. As a control, Figure 4A, lane 19 shows that the P3 (C147A) is incapable of autonomous RNA replication.

The translation results for the P3 (C147A) complementation experiment are displayed in **Figure 4B**. Here, [35S]methionine-labeled translations from the corresponding reactions shown in Figure 4A, was carried out as in Figure 2B with and without 5'-NCR-P3 (C147A). The data shown in lane 19 confirm that P3 (C147A) is incapable of cis-cleavage, indicated by the presence of unprocessed P3 precursor protein and the absence of cleavage products. Consistent with the above hypotheses, the proteolytically inactive 3CD (C147A) protein does not process the VP0-VP3 precursor protein that accumulates from the inefficiency of the CPP, PPC, CPC, and PCC chimeras to cleave at the VP0/VP3 junction. Comparing the levels of VP0-VP3 in lanes 5 and 6, one might conclude that additional processing of this precursor polypeptide by 3CD (C147A) occurred. However, this is most likely an effect of a sample loading error (compare levels of 3CD and VP1 polypeptides in lanes 5 and 6). Surprisingly, one of the chimeras (CCC) showed a bona fide rescue of VP0-VP3 processing (compare lanes 17 and 18), as demonstrated by a reduction in the amount of VP0-VP3 and an appearance of VP0 and VP3 in the presence of P3 (C147A). This rather unexpected result suggests that an inactive 3CD (C147A) polypeptide can enhance the ability of a deficient 3CD molecule [*e.g.*, 3CD (CCC)] to process the VP0/VP3 junction. This could occur through multimerization of two defective 3CD polypeptides to form an active complex or by 3CD (C147A) enhancing the recognition of the Q-G dipeptide within the VP0-VP3 precursor polypeptide by binding and recruiting 3CD (CCC) to the site of proteolytic cleavage.

Translation and RNA replication of PV1/CVB3 transcripts in the presence of P3 (μ 61)

RNA: As discussed above, P3 (wt), P3 (C147A), and P3 (R13N) are likely providing a source of wild type 3D polymerase, generated via a cis or trans cleavage event within P3, to RNA replication complexes that assemble around full length transcript RNAs. The inventors therefore wanted to carry out RNA replication complementation experiments utilizing a P3 precursor polypeptide that yields an inactive polymerase. This experiment utilizes a co-translating 5'-NCR-P3 (μ 61) RNA (see Figure 1C), containing a K61L mutation in the 3D polymerase that renders it incapable of RNA chain elongation.

Figure 5A shows the RNA replication results in the presence and absence of this co-translating RNA. Here, RNA replication carried out as described in for Figure 2A, utilizing a 5'-NCR-P3 (μ 61) transcript in each co-translation reaction (indicated with a plus sign). The autoradiogram shown below is a longer exposure of the same gel. Unlike the other forms of P3 tested, P3 (μ 61) was unable to rescue positive-strand RNA synthesis when incubated with full length CPP chimeric RNA, although a detectable increase in negative-strand RNA synthesis was observed (compare lanes 5 and 6). This demonstrates that in previous experiments with this chimera, wild type 3D provided in trans was the primary source of polymerase that allowed positive-strand RNA synthesis to occur. The increase in negative-strand RNA synthesis in the absence of new positive-strands suggests that P3 (μ 61) proteins are capable of recruiting active CPP polymerase to sites of negative-strand RNA synthesis initiation. These data provide additional evidence that the viral proteins that form complexes to initiate negative-strand RNA synthesis are different, in part, from those that assemble for the synthesis of positive-strands.

The PCP and CCP chimeras were equally responsive to the presence of P3 (μ 61) and showed an increase in both negative- and positive-strand RNA synthesis (compare lane 7 to 8 and 13 to 14). These data indicate that the PCP and CCP chimeric polymerases are equally capable of interacting with P3 (μ 61) proteins in the formation of RNA synthesis initiation complexes. However, given the results with CPP (lanes 5 and 6), the inventors predict that the increase in positive-strand RNA generated by these two chimeras is primarily the result of an increase in negative-strand RNA synthesis in the presence of P3 (μ 61) proteins, thus providing significant quantities of newly-synthesized templates for positive-strand RNA synthesis. In contrast, chimeras PPC and CPC were completely unresponsive to rescue with P3 (μ 61) (lanes 9-12), whereas they were rescued with P3 (wt) (Figure 2A), P3 (R13N)

(Figure 3A), and to a limited extent P3 (C147A) (Figure 4A). These observations, combined with the fact that P3 ($\mu 61$) cannot provide active polymerase molecules, suggest that the PPC and CPC 3D polymerases could be enzymatically defective. Finally, chimeras PCC and CCC were also unresponsive to rescue by P3 ($\mu 61$) (compare lane 15 to 16 and 17 to 18). Since earlier results with these chimeras utilizing P3 (R13N) indicate a deficiency in protein-protein interactions with complementing polypeptides, conclusive conclusions regarding the activity of the PCC and CCC chimeric polymerases themselves can not be drawn on the basis of the current findings. The rescue phenotypes of these two chimeras may simply be a result of an inability of chimeric 3D polymerase molecules to be recruited by P3 ($\mu 61$) polypeptides to RNA replication complexes.

The translation results for the P3 ($\mu 61$) rescue experiment are shown in **Figure 5B**. Here, [35S]methionine-labeled translations from the corresponding reactions shown in Figure 5A, was carried out as in Figure 2B with and without 5'-NCR-P3 ($\mu 61$). Interestingly, the presence of 3CD ($\mu 61$) provided by the P3 ($\mu 61$) co-translating RNA (even numbered lanes 4-18) did not result in the complete processing of VP0-VP3 precursor polypeptide in trans (lanes 2-18, compare even to odd numbered lanes). Earlier results have shown that point mutations within the polymerase sub-domain of 3CD can reduce the ability of 3CD to recognize the VP0/VP3 junction. These results suggest that the K61L mutation present in context of the 3CD ($\mu 61$) polypeptide provided in trans has this same effect. Furthermore, in the absence of complete VP0-VP3 processing, an increase in RNA synthesis is observed in the presence of P3 ($\mu 61$) for chimeras CPP, PCP and CCP (see *e.g.*, Figure 5A, compare lane 5 to 6, 7 to 8 and 13 to 14). Combined with results presented previously in this work, these findings confirm that complete VP0-VP3 processing is not required for efficient RNA synthesis.

3Dpol *in vitro* elongation assays: Data presented herein strongly suggest alterations to 3D RNA polymerase enzymatic activities in each of the PV1/CVB3 chimeras. This is supported by both the dramatic differences in autonomous replication (compared to wild type), as well as a differential responsiveness of each chimera to P3 complementation. To test this possibility, the inventors assayed directly for RNA chain elongation activity encoded by each of the PV1/CVB3 3D polymerases using a method previously described. First, *in vitro* translation reactions were carried out with wild type PV1 RNA or PV1/CVB3 transcripts. Then, a portion of each reaction was used as a source of 3D RNA polymerase to catalyze the

elongation of RNA chains from an oligo(U)-primed PV1 virion RNA template in the presence of radiolabeled CTP (see above). Aliquots from each reaction were removed at specific times during a 60 minute reaction, and the amount of incorporated nucleotide was measured and used as an indication of RNA polymerase elongation activity. The results of this experiment are shown in **Figure 6**. Here, wild type and PV1/CVB3 transcripts were translated *in vitro*, providing a source of 3D RNA polymerase used to elongate nascent RNA chains from an oligo(U)-primed vRNA template as described below. Aliquots at 0, 15, 30, and 60 minutes were counted, and after subtracting background levels of radioactivity, the data were plotted. The graph shown in Figure 6 displays representative data from two independent experiments.

Wild type polymerase was capable of incorporating significant amounts of radiolabeled CTP during the time course experiment. As expected, chimeras CPP, PCP, and CCP, each previously shown to be capable of autonomous RNA replication, have similar elongation activities, albeit slightly reduced when compared to wild type polymerase. Interestingly, the PPC chimeric polymerase is also able to elongate from the oligo(U)-primed vRNA primed template. This strengthens the inventors' earlier conclusion that 3D/3CD (PPC) is deficient in protein-protein contacts required for the formation of RNA synthesis complexes (and could also indicate a deficiency in RNA synthesis initiation rather than elongation). The inventors also hypothesize that the CPC chimera is deficient in similar protein-protein contacts, exhibited by its reduced ability to be complemented in trans by all forms of P3 tested. The results in Figure 6 indicate that an additional defect exists in RNA elongation activity as well. Finally, chimeras PCC and CCC displayed the lowest levels of elongation activity compared to the other constructs, reflecting the observation that these two chimeras were almost completely unresponsive to RNA synthesis complementation by the P3 constructs used herein.

A summary of the P3 complementation data is displayed in **Figure 7**. Here, the 3D polymerase composition of each full-length chimeric transcript tested is indicated at the top of the figure. Shown below the chimeras is a qualitative assessment (indicated by high, low, or none) of the ability of each to replicate autonomously or to be complemented in trans by wild type or genetically-altered P3 protein (left side of panel). Each is shown as negative-strand RNA synthesis over positive-strand RNA synthesis (see bottom of figure). Based on a comparison to RNA synthesis levels observed for wild type PV1 transcript, all chimeras capable of synthesizing RNA in the absence of complementing P3 protein were given the

designation "low." For the complementation results with and without P3 protein, the designation "high" was assigned to RNA replication levels estimated to be at least 5-fold greater than those observed in the absence of P3 protein. "Low" indicates an increase in RNA levels approximately 2 to 4 fold over those observed without P3, whereas "none"

5 indicates no detectable increase in RNA synthesis in the presence of P3. Based upon the differential rescue of the 3D polymerase chimeras, the inventors divided these chimeras into three functional groups. Members of one group include CPP, PCP, and CCP, which encode an active 3D RNA polymerase, as evidenced by autonomous RNA replication (albeit at low levels). A second group includes PPC and CPC, which encode various 3D RNA polymerase
10 molecules with reduced elongation activity, unable to efficiently assemble RNA replication complexes. Support for this conclusion comes from the polymerase elongation assays, the lack of autonomous RNA replication, the inability to be complemented by a P3 precursor protein harboring an enzymatically inactive 3D polymerase, and molecular modeling studies that predict the disruption of one or more protein-protein interfaces shown to be important for
15 3D RNA polymerase activity. Members of the final group that includes the chimeras PCC and CCC, appear to be incapable of participating in protein-protein interactions critical for RNA replication and encode RNA polymerases with the lowest levels of RNA synthesis activity detected in the elongation assays (see also Figure 6).

Attempts to rescue defects in RNA synthesis with a wild type P3 precursor protein
20 resulted in an increase in RNA replication for each of the seven chimeras tested. The rescue of chimeras that do not replicate autonomously (here: PPC, CPC, PCC, and CCC) suggests that either wild type proteins provided in trans can form complexes with viral RNA independent of chimeric binding partners, or that there are protein-protein contacts that allow recruitment of polypeptides provided in trans to sites of RNA synthesis initiation. The
25 importance of protein-protein contacts in recruitment of viral polypeptides to active RNA replication complexes is underscored by the results with P3 (R13N), since this form of P3 yields a 3CD molecule incapable of binding cloverleaf RNA but capable of rescuing replication in some of the chimeras. These results also indicate that sequences within the amino-terminal and carboxy-terminal one-third of the 3D polymerase (or 3D domain of the
30 3CD polypeptide) are necessary for productive interactions with proteins provided in trans, which could involve 3D-3D, 3D-3CD, 3CD-3CD, 3D-3AB, or 3CD-3AB contacts. Earlier

findings regarding defective 3CD (CPC) and 3CD (PPC) protein-protein interactions appear to support this hypothesis.

A P3 precursor yielding an inactive polymerase ($\mu 61$) was able to increase negative-strand RNA synthesis, but only in those chimeras capable of autonomous RNA replication (CPP, PCP, and CCP). This suggests that a precursor to the polymerase (here: 3CD) can recruit active chimeric polymerase molecules (provided in cis) to sites of negative-strand RNA synthesis initiation, possibly via 3CD-3CD or 3CD-3D interactions mediated by 3D polymerase domain contacts. There may be a mechanism to favor assembly of protein-protein and protein-RNA complexes which involve polypeptides provided in cis. However, when polymerase or polymerase precursor polypeptides provided in cis are incapable of forming one or more complexes, proteins provided in trans are no longer kinetically excluded and may participate in replication complex assembly. This is evidenced by complementation of RNA synthesis of the PPC, CPC, PCC, and CCC chimeras (incapable of replicating on their own, and therefore of assembling one or more cis-dependent complexes) with a wild type P3 precursor. For CPP, the chimeric proteins provided in cis are unable to form complexes necessary for positive-strand RNA synthesis, making the replication proteins provided in trans the only source of active polymerase used for this process. When active polymerase is not provided in trans, as is the case for the P3 ($\mu 61$) construct, no rescue of positive-strand RNA synthesis was observed (Figure 5A, compare lanes 5 and 6).

For two of the chimeras incapable of autonomous RNA replication (PPC and CPC), one can draw significant conclusions regarding the source of the 3D RNA polymerase provided to RNA synthesis complexes. Previously published biochemical data indicated that certain chimeric 3CD polypeptides are capable of forming ribonucleoprotein complexes with the poliovirus cloverleaf RNA and host protein PCBP2 with an efficiency greater than that of wt 3CD. Since neither PPC nor CPC replicate in the absence of a P3 sub-genomic RNA, these two chimeras may bind viral RNA in cis, but are deficient in other protein-protein interactions necessary for RNA synthesis initiation separate from those involving PCBP2. Such findings provide an explanation for their differential responses to complementation using the P3 (R13N) and P3 (C147A) constructs, both of which yield sufficient levels of wild type 3D RNA polymerase. Taken together, these results suggest a kinetically-favored assembly of highest-affinity ribonucleoprotein complexes in cis.

Therefore, the results from the complementation analysis of negative- and positive-strand RNA synthesis presented herein yield insights into possible mechanisms utilized for the assembly of strand-specific RNA replication complexes. These insights include: (i) higher-order complexes containing 3CD molecules that could serve as donors of 3D RNA polymerase favor assembly with viral proteins provided in cis, (ii) protein-protein interactions between viral proteins in cis and those in trans can facilitate the recruitment of helper functions derived from proteins that are, by themselves, deficient in binding viral RNA, and (iii) protein complexes that assemble for negative- versus positive-strand RNA synthesis are not equally capable of recruiting and/or utilizing functional P3 proteins provided in trans, thereby inhibiting the progression of RNA synthesis when such proteins are limiting or defective. These differential effects are mediated, in part, by specific domains within proteins harboring 3D amino acid sequences. However, the roles that these domains have during specific steps of RNA replication (VPg uridylylation, interactions with host proteins, RNA chain elongation, putative subunit exchange between strand-specific RNA synthesis complexes) remain to be determined.

References

Unless stated otherwise, all publications and patents are incorporated by reference herein. Where a definition or use of a term in a reference, which is incorporated by reference herein is inconsistent or contrary to the definition of that term provided herein, the definition of that term provided herein applies and the definition of that term in the reference does not apply.

Andino, R., G. E. Rieckhof, P. L. Achacoso, and D. Baltimore. 1993. Poliovirus RNA synthesis utilizes an RNP complex formed around the 5'-end of viral RNA. EMBO J. 12:3587-3598.

Andino, R., G. E. Rieckhof, and D. Baltimore. 1990. A functional ribonucleoprotein complex forms around the 5' end of poliovirus RNA. Cell 63:369-380.

Barton, D. J., E. P. Black, and J. B. Flanagan. 1995. Complete replication of poliovirus in vitro: preinitiation RNA replication complexes require soluble cellular factors for the synthesis of VPg-linked RNA. J. Virol. 69:5516-5527.

Barton, D. J. and J. B. Flanagan. 1993. Coupled translation and replication of poliovirus RNA in vitro: synthesis of functional 3D polymerase and infectious virus. *J.Virol.* 67:822-831.

Barton, D. J., B. J. O'Donnell, and J. B. Flanagan. 2001. 5' cloverleaf in poliovirus RNA is a cis-acting replication element required for negative-strand synthesis. *EMBO J.* 20:1439-1448.

Bell, Y. C., B. L. Semler, and E. Ehrenfeld. 1999. Requirements for RNA replication of a poliovirus replicon by coxsackievirus B3 RNA polymerase. *J.Virol.* 73:9413-9421.

Bernstein, H. D., P. Sarnow, and D. Baltimore. 1986. Genetic complementation among poliovirus mutants derived from an infectious cDNA clone. *J.Virol.* 60:1040-1049.

Bienz, K., D. Egger, and L. Pasamontes. 1987. Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography. *Virology* 160:220-226.

Blair, W. S., T. B. Parsley, H. P. Bogerd, J. S. Towner, B. L. Semler, and B. R. Cullen. 1998. Utilization of a mammalian cell-based RNA binding assay to characterize the RNA binding properties of picornavirus 3C proteinases. *RNA.* 4:215-225.

Borman, A. M., R. Kirchweiger, E. Ziegler, R. E. Rhoads, T. Skern, and K. M. Kean. 1997. eIF4G and its proteolytic cleavage products: effect on initiation of protein synthesis from capped, uncapped, and IRES-containing mRNAs. *RNA* 3:186-196.

Cao, X. and E. Wimmer. 1995. Intragenomic complementation of a 3AB mutant in dicistronic polioviruses. *Virology* 209:315-326.

Charini, W. A., C. C. Burns, E. Ehrenfeld, and B. L. Semler. 1991. trans rescue of a mutant poliovirus RNA polymerase function. *J.Virol.* 65:2655-2665.

Cho, M. W., N. Teterina, D. Egger, K. Bienz, and E. Ehrenfeld. 1994. Membrane rearrangement and vesicle induction by recombinant poliovirus 2C and 2BC in human cells. *Virology* 202:129-145.

Cornell, C. T., R. Perera, J. E. Brunner, and B. L. Semler. 2004. Strand-specific RNA synthesis determinants in the RNA-dependent RNA polymerase of poliovirus. *J Virol.* 78:4397-407.

Cornell, C. T., J. E. Brunner, and B. L. Semler. 2004. Differential rescue of poliovirus RNA replication functions by genetically modified RNA polymerase precursors. *J Virol.* 78:13007-13018.

Cornell, C. T. and B. L. Semler. 2002. Subdomain specific functions of the RNA polymerase region of poliovirus 3CD polypeptide. *Virology* 298:200-213.

Deitz, S. B., D. A. Dodd, S. Cooper, P. Parham, and K. Kirkegaard. 2000. MHC I-dependent antigen presentation is inhibited by poliovirus protein 3A. *Proc.Natl.Acad.Sci.U.S.A* 97:13790-13795.

Dewalt, P. G. and B. L. Semler . 1989. Molecular biology and genetics of poliovirus protein processing, p. 73-93. In B. L. Semler and E. Ehrenfeld (ed.), *Molecular genetic aspects of picornavirus infection and detection*. American society for microbiology, Washington D.C.

Doedens, J. R., T. H. J. Giddings, and K. Kirkegaard. 1997. Inhibition of endoplasmic reticulum-to-Golgi traffic by poliovirus protein 3A: genetic and ultrastructural analysis. *J.Virol.* 71:9054-9064.

Doedens, J. R. and K. Kirkegaard. 1995. Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A. *EMBO J.* 14:894-907.

Egger, D., N. Teterina, E. Ehrenfeld, and K. Bienz. 2000. Formation of the poliovirus replication complex requires coupled viral translation, vesicle production, and viral RNA synthesis. *J.Virol.* 74:6570-6580.

Etchison, D., S. C. Milburn, I. Edery, N. Sonenberg, and J. W. Hershey. 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. *J.Biol.Chem.* 257:14806-14810.

Giachetti, C., S. S. Hwang, and B. L. Semler. 1992. cis-acting lesions targeted to the hydrophobic domain of a poliovirus membrane protein involved in RNA replication. *J.Virol.* 66:6045-6057.

5 Gustin, K. E. and P. Sarnow. 2001. Effects of poliovirus infection on nucleocytoplasmic trafficking and nuclear pore complex composition. *EMBO J.* 20:240-249.

Hanecak, R., B. L. Semler, C. W. Anderson, and E. Wimmer. 1982. Proteolytic processing of poliovirus polypeptides: antibodies to polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs. *Proc.Natl.Acad.Sci.U.S.A.* 79:3973-3977.

10 Harris, K. S., S. R. Reddigari, M. J. Nicklin, T. Hammerle, and E. Wimmer. 1992. Purification and characterization of poliovirus polypeptide 3CD, a proteinase and a precursor for RNA polymerase. *J.Virol.* 66:7481-7489.

Harris, K. S., W. Xiang, L. Alexander, W. S. Lane, A. V. Paul, and E. Wimmer. 1994. Interaction of poliovirus polypeptide 3CDpro with the 5' and 3' termini of the poliovirus genome. Identification of viral and cellular cofactors needed for efficient binding.
15 *J.Biol.Chem.* 269:27004-27014.

Herold, J. and R. Andino. 2000. Poliovirus requires a precise 5' end for efficient positive-strand RNA synthesis. *J.Virol.* 74:6394-6400.

Herold, J. and R. Andino. 2001. Poliovirus RNA replication requires genome circularization through a protein-protein bridge. *Mol.Cell* 7:581-591.

20 Hobson, S. D., E. S. Rosenblum, O. C. Richards, K. Richmond, K. Kirkegaard, and S. C. Schultz. 2001. Oligomeric structures of poliovirus polymerase are important for function. *EMBO J.* 20:1153-1163.

Hope, D. A., S. E. Diamond, and K. Kirkegaard. 1997. Genetic dissection of interaction between poliovirus 3D polymerase and viral protein 3AB. *J.Virol.* 71:9490-9498.

25 Johnson, K. L. and P. Sarnow. 1991. Three poliovirus 2B mutants exhibit noncomplementable defects in viral RNA amplification and display dosage-dependent dominance over wild-type poliovirus. *J.Virol.* 65:4341-4349.

Jore, J., B. De Geus, R. J. Jackson, P. H. Pouwels, and B. E. Enger-Valk. 1988. Poliovirus protein 3CD is the active protease for processing of the precursor protein P1 in vitro. *J.Gen.Virol.* 69:1627-1636.

Jurgens, C. and J. B. Flanagan . 2003. Initiation of poliovirus negative-strand RNA
5 synthesis requires precursor forms of p2 proteins. *J.Virol.* 77:1075-1083.

Krausslich, H. G., M. J. Nicklin, H. Toyoda, D. Etchison, and E. Wimmer. 1987. Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220. *J.Virol.* 61:2711-2718.

Lawson, M. A. and B. L. Semler . 1991. Poliovirus thiol proteinase 3C can utilize a
10 serine nucleophile within the putative catalytic triad. *Proc.Natl.Acad.Sci.U.S.A.* 88:9919-9923.

Leong, L. E., C. T. Cornell, and B. L. Semler. 2002. Processing Determinants and Functions of Cleavage Products of Picornavirus Polyproteins, p. 187-197. In B. L. Semler and E. Wimmer (ed.), *Molecular Biology of Picornaviruses*. ASM Press.

15 Li, J. P. and D. Baltimore. 1988. Isolation of poliovirus 2C mutants defective in viral RNA synthesis. *J.Virol.* 62:4016-4021.

Lloyd, R. E., H. G. Jense, and E. Ehrenfeld. 1987. Restriction of translation of capped mRNA in vitro as a model for poliovirus-induced inhibition of host cell protein synthesis: relationship to p220 cleavage. *J.Virol.* 61:2480-2488.

20 Molla, A., A. V. Paul, and E. Wimmer. 1991. Cell-free, de novo synthesis of poliovirus. *Science* 254:1647-1651.

Novak, J. E. and K. Kirkegaard . 1994. Coupling between genome translation and replication in an RNA virus. *Genes Dev.* 8:1726-1737.

Parsley, T. B., C. T. Cornell, and B. L. Semler. 1999. Modulation of the RNA binding
25 and protein processing activities of poliovirus polypeptide 3CD by the viral RNA polymerase domain. *J.Biol.Chem.* 274:12867-12876.

Parsley, T. B., J. S. Towner, L. B. Blyn, E. Ehrenfeld, and B. L. Semler. 1997. Poly (rC) binding protein 2 forms a ternary complex with the 5'- terminal sequences of poliovirus RNA and the viral 3CD proteinase. *RNA*. 3:1124-1134.

Pata, J. D., S. C. Schultz, and K. Kirkegaard. 1995. Functional oligomerization of
5 poliovirus RNA-dependent RNA polymerase. *RNA*. 1:466-477.

Paul, A. V., E. Rieder, D. W. Kim, J. H. van Boom, and E. Wimmer. 2000. Identification of an RNA hairpin in poliovirus RNA that serves as the primary template in the in vitro uridylylation of VPg. *J.Virol.* 74:10359-10370.

Paul, A. V., J. H. van Boom, D. Filippov, and E. Wimmer. 1998. Protein-primed RNA
10 synthesis by purified poliovirus RNA polymerase. *Nature* 393:280-284.

Richards, O. C., S. Baker, and E. Ehrenfeld. 1996. Mutation of lysine residues in the nucleotide binding segments of the poliovirus RNA-dependent RNA polymerase. *J.Virol.* 70:8564-8570.

Rieder, E., A. V. Paul, D. W. Kim, J. H. van Boom, and E. Wimmer. 2000. Genetic
15 and biochemical studies of poliovirus cis-acting replication element cre in relation to VPg uridylylation. *J.Virol.* 74:10371-10380.

Semler, B. L., C. W. Anderson, N. Kitamura, P. G. Rothberg, W. L. Wishart, and E. Wimmer. 1981. Poliovirus replication proteins: RNA sequence encoding P3-1b and the sites of proteolytic processing. *Proc.Natl.Acad.Sci.U.S.A* 78:3464-3468.

20 Teterina, N. L., A. E. Gorbalenya, D. Egger, K. Bienz, and E. Ehrenfeld. 1997. Poliovirus 2C protein determinants of membrane binding and rearrangements in mammalian cells. *J.Virol.* 71:8962-8972.

Teterina, N. L., W. D. Zhou, M. W. Cho, and E. Ehrenfeld. 1995. Inefficient complementation activity of poliovirus 2C and 3D proteins for rescue of lethal mutations.
25 *J.Virol.* 69:4245-4254.

Todd, S., J. S. Towner, D. M. Brown, and B. L. Semler. 1997. Replication-competent picornaviruses with complete genomic RNA 3' noncoding region deletions. *J.Virol.* 71:8868-8874.

Towner, J. S., M. M. Mazanet, and B. L. Semler. 1998. Rescue of defective poliovirus RNA replication by 3AB-containing precursor polyproteins. *J.Virol.* 72:7191-7200.

Towner, J. S., H. H. Roehl, S. Todd, T. P. Parsley, T. V. Ho, and B. L. Semler. 1996. Complex formation and template recognition in poliovirus RNA replication, p. 3-22. In S.Jameel and E.K.Wagner (ed.), *Current Developments in Animal Virology*. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi.

Van Dyke, T. A. and J. B. Flanagan. 1980. Identification of poliovirus polypeptide P63 as a soluble RNA- dependent RNA polymerase. *J.Virol.* 35:732-740.

Walter, B. L., T. B. Parsley, E. Ehrenfeld, and B. L. Semler. 2002. Distinct poly(rC) binding protein KH domain determinants for poliovirus translation initiation and viral RNA replication. *J.Virol.* 76:12008-12022.

Weidman, M. K., P. Yalamanchili, B. Ng, W. Tsai, and A. Dasgupta. 2001. Poliovirus 3C protease-mediated degradation of transcriptional activator p53 requires a cellular activity. *Virology* 291:260-271.

Xiang, W., A. Cuconati, D. Hope, K. Kirkegaard, and E. Wimmer. 1998. Complete protein linkage map of poliovirus P3 proteins: interaction of polymerase 3Dpol with VPg and with genetic variants of 3AB. *J.Virol.* 72:6732-6741.

Yalamanchili, P., R. Banerjee, and A. Dasgupta. 1997. Poliovirus-encoded protease 2APro cleaves the TATA-binding protein but does not inhibit host cell RNA polymerase II transcription in vitro. *J.Virol.* 71:6881-6886.

Yalamanchili, P., U. Datta, and A. Dasgupta. 1997. Inhibition of host cell transcription by poliovirus: cleavage of transcription factor CREB by poliovirus-encoded protease 3Cpro. *J.Virol.* 71:1220-1226.

Yalamanchili, P., K. Weidman, and A. Dasgupta. 1997. Cleavage of transcriptional activator Oct-1 by poliovirus encoded protease 3Cpro. *Virology* 239:176-185.

Ypma-Wong, M. F., P. G. Dewalt, V. H. Johnson, J. G. Lamb, and B. L. Semler. 1988. Protein 3CD is the major poliovirus proteinase responsible for cleavage of the P1 capsid precursor. *Virology* 166:265-270.

Thus, specific embodiments and applications of viral chimera compositions and methods have been disclosed. It should be apparent, however, to those skilled in the art that many more modifications besides those already described are possible without departing from the inventive concepts herein. The inventive subject matter, therefore, is not to be restricted
5 except in the spirit of the appended claims. Moreover, in interpreting both the specification and the claims, all terms should be interpreted in the broadest possible manner consistent with the context. In particular, the terms "comprises" and "comprising" should be interpreted as referring to elements, components, or steps in a non-exclusive manner, indicating that the referenced elements, components, or steps may be present, or utilized, or combined with other
10 elements, components, or steps that are not expressly referenced.

CLAIMS

What is claimed is:

1. A test system comprising:

5 a plurality of chimeric nucleic acids in which a first nucleic acid portion is from a first virus of a picornavirus family and in which a second nucleic acid portion is from a second virus of the picornavirus family, respectively;

wherein each of the second portions is a sub-allelic portion of one viral gene of the second virus;

10 a second nucleic acid selected from the group consisting of a nucleic acid encoding a wild-type P3 polypeptide, a P3 polypeptide that is defective in cloverleaf binding, a P3 polypeptide that is defective in protein processing, and a P3 polypeptide that has defective 3D polymerase function; and

15 wherein the sub-allelic portions are selected such that a first and a second of the chimeric nucleic acids exhibit a distinct replication characteristic when co-expressed with the second nucleic acid.

2. The test system of claim 1 wherein the replication characteristic is characteristic for a negative strand RNA synthesis and selected from the group consisting of autonomous RNA replication, complementation by wild-type P3, complementation by P3 defective in cloverleaf binding, complementation by P3 defective in protein processing, and
20 complementation by P3 with defective 3D polymerase.

3. The test system of claim 1 wherein the replication characteristic is characteristic for a positive strand RNA synthesis and selected from the group consisting of autonomous RNA replication, complementation by wild-type P3, complementation by P3 defective in cloverleaf binding, complementation by P3 defective in protein processing, and
25 complementation by P3 with defective 3D polymerase.

4. The test system of claim 1 wherein the viral gene is the 3D gene.

5. The test system of claim 1 wherein the 3D gene has a first, a second, and a third section, and wherein the sub-allelic portion covers the first and optionally the second
30 section.

6. The test system of claim 1 wherein the 3D gene has a first, a second, and a third section, and wherein the sub-allelic portion covers the second and optionally the third section.
7. The test system of claim 1 wherein the 3D gene has a first, a second, and a third section, and wherein the sub-allelic portion covers the first and third section.
8. The test system of claim 1 wherein the system includes at least five distinct chimeric nucleic acids.
9. The test system of claim 1 wherein the plurality of nucleic acids are double stranded DNA.
10. The test system of claim 1 wherein the first virus is a poliovirus and wherein the second virus is a coxsackie virus.
11. The test system of claim 1 further comprising at least one of a nucleic acid encoding a wild-type P3 polypeptide, a P3 polypeptide that is defective in cloverleaf binding, a P3 polypeptide that is defective in protein processing, and a P3 polypeptide that has defective 3D polymerase function.
12. The test system of claim 1 further comprising an instruction that instructs a user to test a pharmaceutically active compound in an *in vitro* system using at least one of the chimeric nucleic acids.
13. A method of providing a test system for test of an antiviral drug, comprising
providing a test system according to claim 1;
providing instructions to express at least one of the chimeric nucleic acids *in vitro* in the presence of an antiviral drug;
providing instructions to optionally co-express at least one nucleic acid that encodes a polypeptide selected from the group consisting of a wild-type P3 polypeptide, a P3 polypeptide that is defective in cloverleaf binding, a P3 polypeptide that is defective in protein processing, and a P3 polypeptide that has defective 3D polymerase function; and

providing instructions to observe *in vitro* synthesis of at least one of a positive strand picornavirus RNA and a negative strand picornavirus RNA.

14. The method of claim 13 further comprising a step of providing instructions to correlate the synthesis of the positive strand or the negative strand picornavirus RNA with a mechanism of antiviral action of the antiviral drug.

15. The method of claim 13 further comprising a step of providing instructions to translate antiviral activity of the antiviral drug to another virus species based on the observation of *in vitro* synthesis of the positive strand or negative picornavirus RNA.

16. The method of claim 13 wherein the step of providing instructions to express at least one of the chimeric nucleic acids *in vitro* in the presence of the antiviral drug is independently performed from the step of providing the test system.

17. The method of claim 13 further comprising a step of providing the at least one nucleic acid that encodes the polypeptide selected from the group consisting of the wild-type P3 polypeptide, the P3 polypeptide that is defective in cloverleaf binding, the P3 polypeptide that is defective in protein processing, and the P3 polypeptide that has the defective 3D polymerase function.

18. A method of testing an antiviral drug, comprising:

expressing a chimeric nucleic acid *in vitro* in the presence of an antiviral drug;

wherein a first nucleic acid portion of the chimeric nucleic acid is from a first virus of a picornavirus family, and wherein a second nucleic acid portion of the chimeric nucleic acid is from a second virus of the picornavirus family;

wherein the second nucleic acid portion encodes at least a portion of a polymerase gene; and

measuring synthesis of at least one of a positive strand picornavirus RNA and a negative strand picornavirus RNA.

19. The method of claim 18 further comprising a step of co-expressing at least one nucleic acid that encodes a polypeptide selected from the group consisting of a wild-type P3 polypeptide, a P3 polypeptide that is defective in cloverleaf binding, a P3 polypeptide

that is defective in protein processing, and a P3 polypeptide that has defective 3D polymerase function.

20. The method of claim 19 wherein the first virus is a poliovirus, and wherein the second virus is a coxsackie virus.

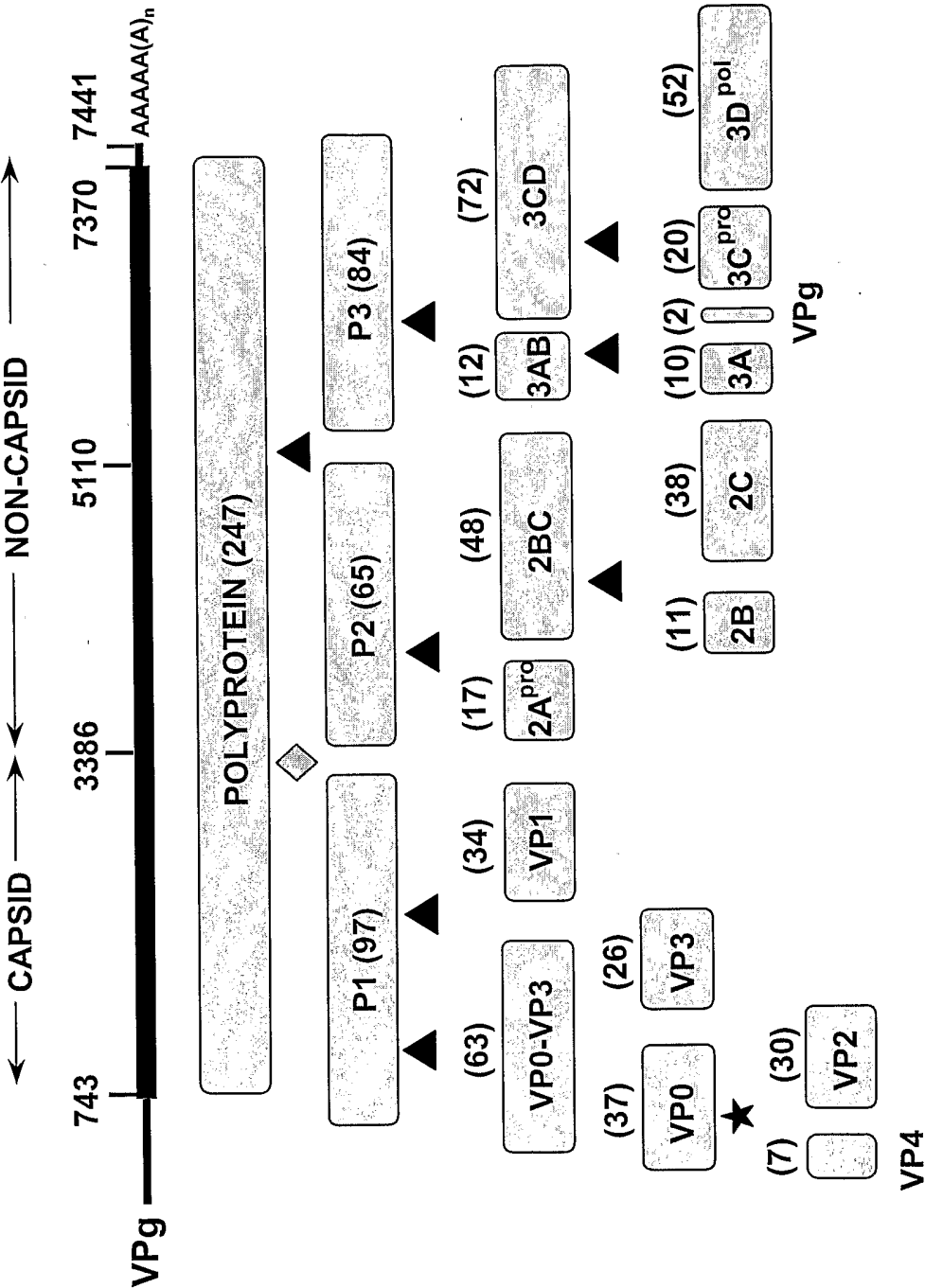


Figure 1A

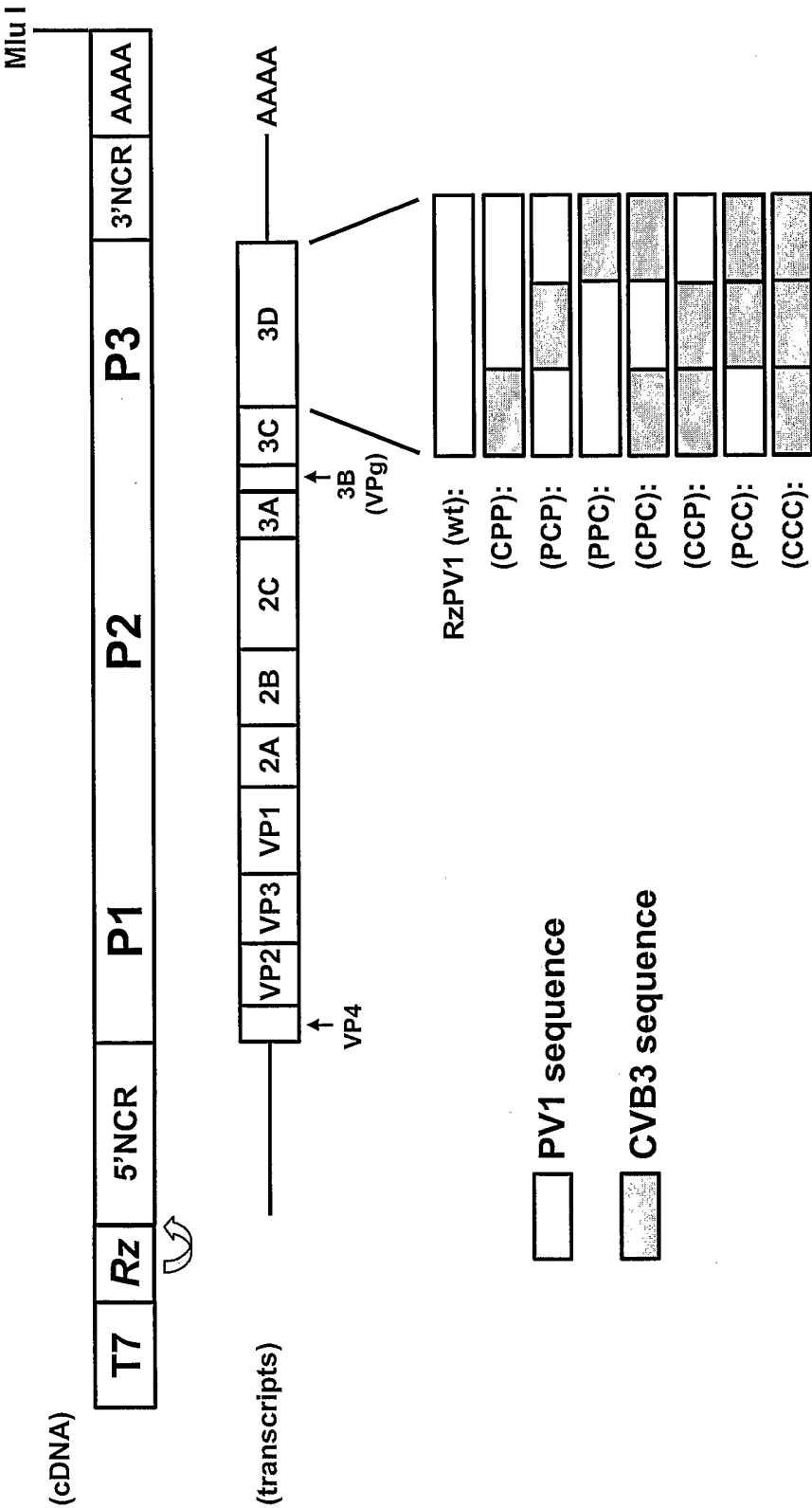


Figure 1B

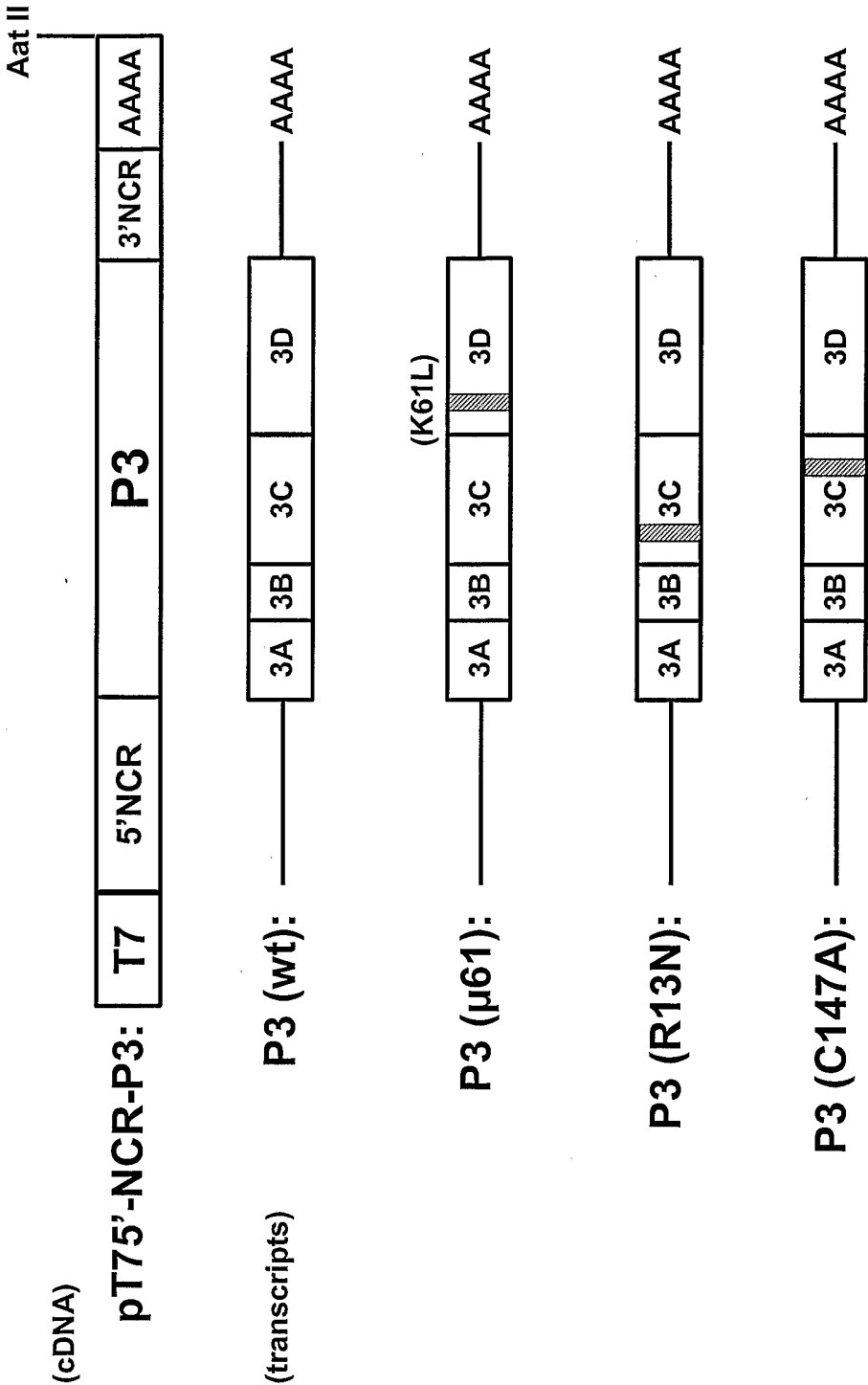


Figure 1C

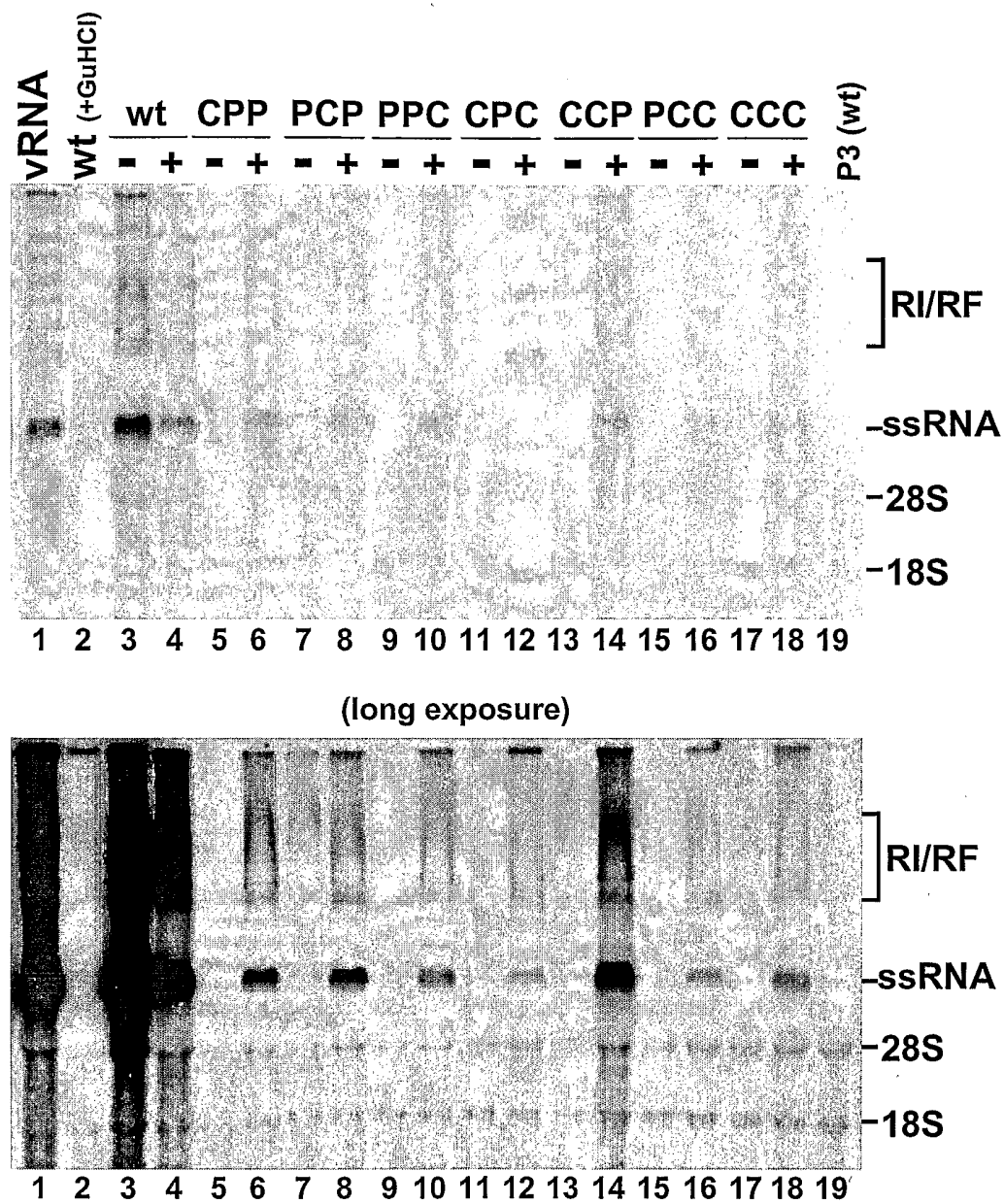


Figure 2A

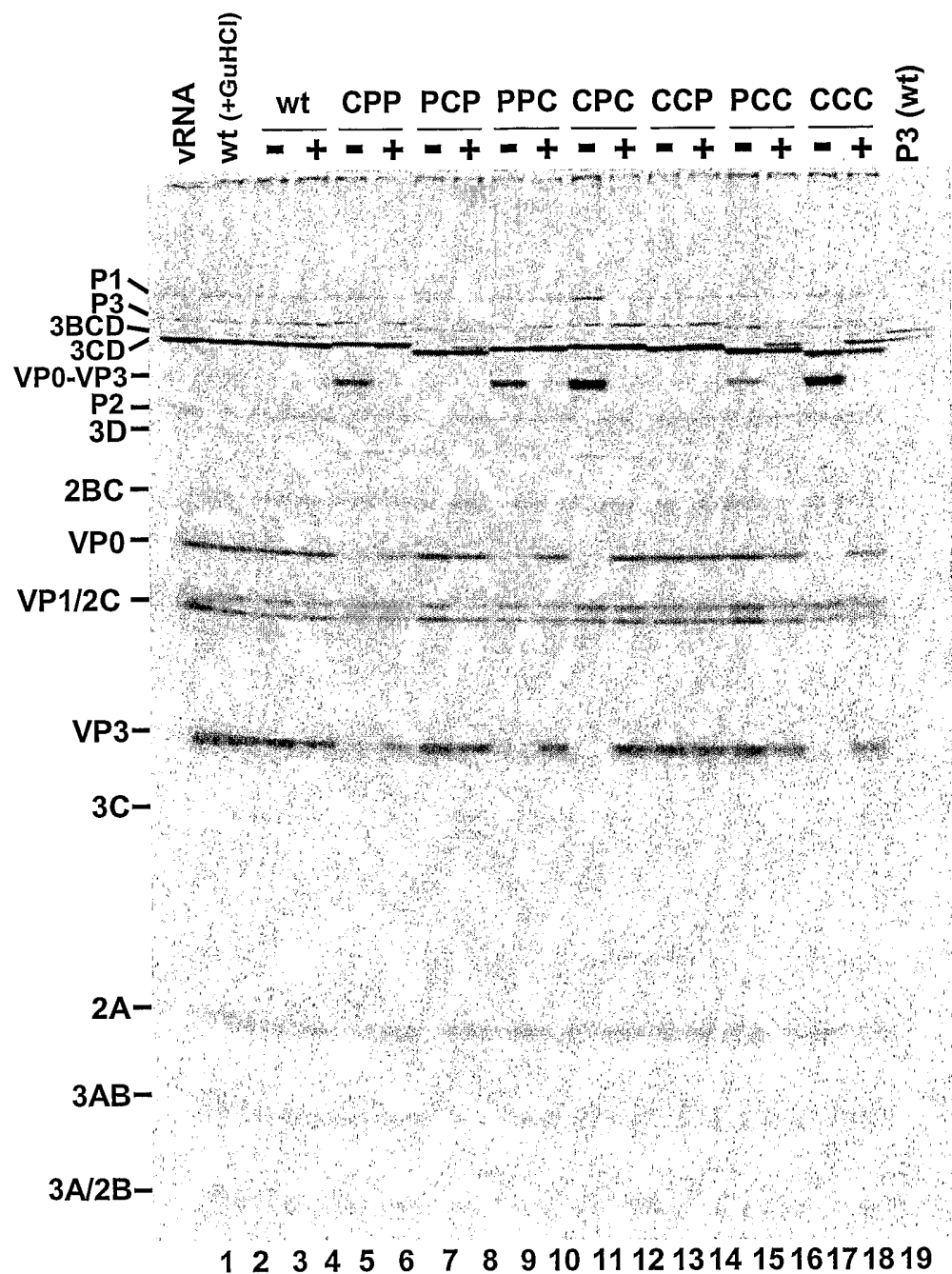


Figure 2B

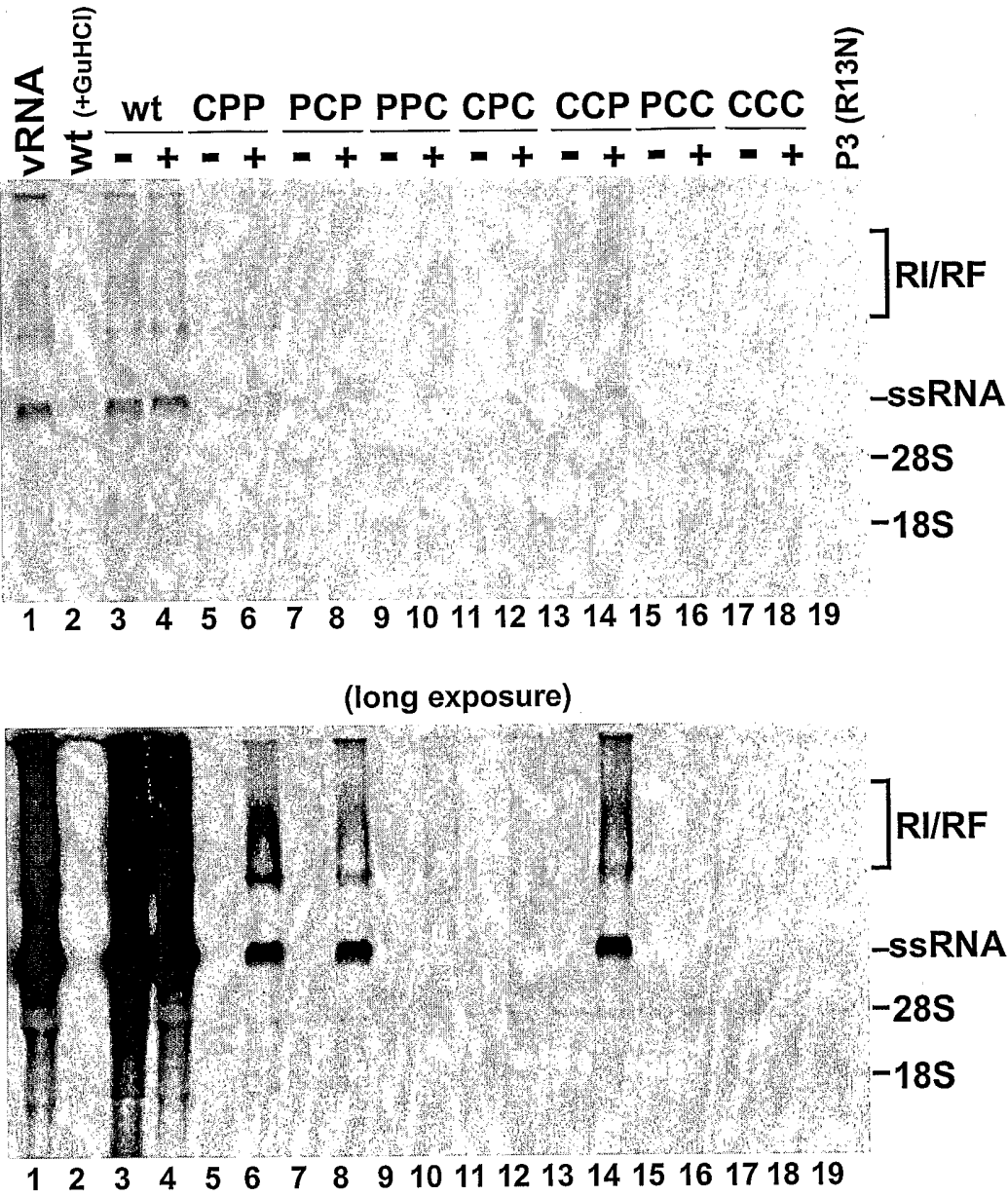


Figure 3A

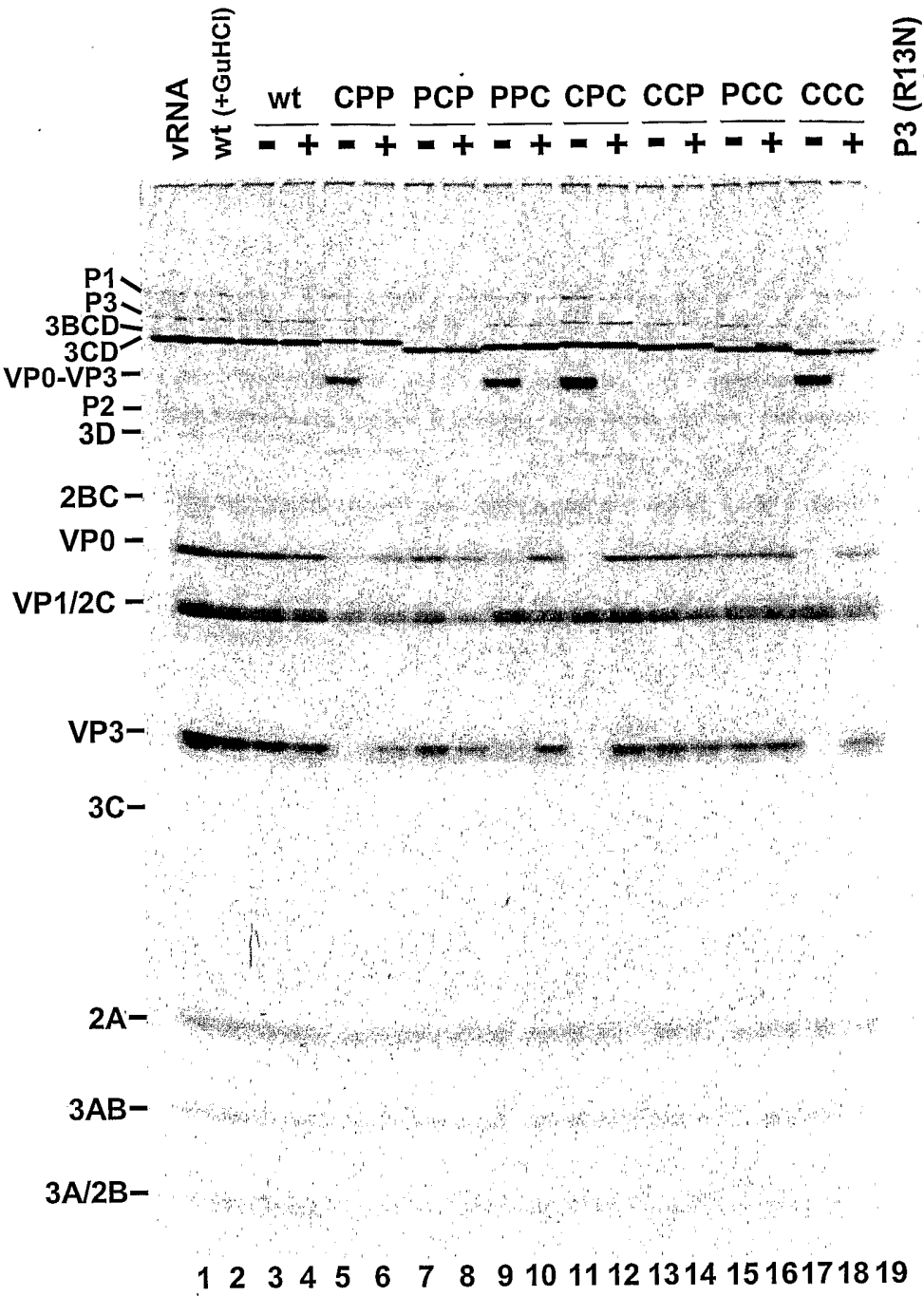


Figure 3B

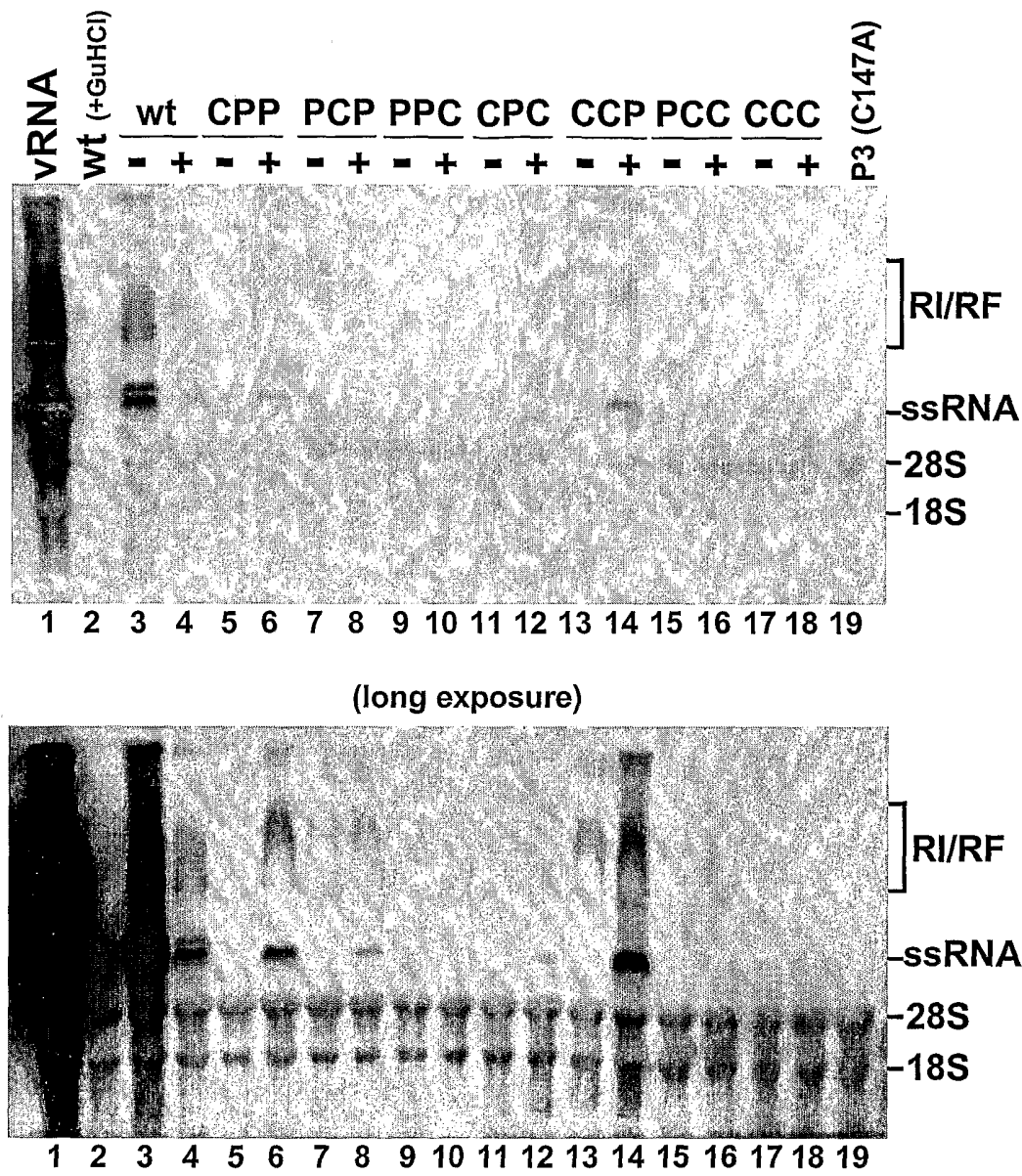


Figure 4A

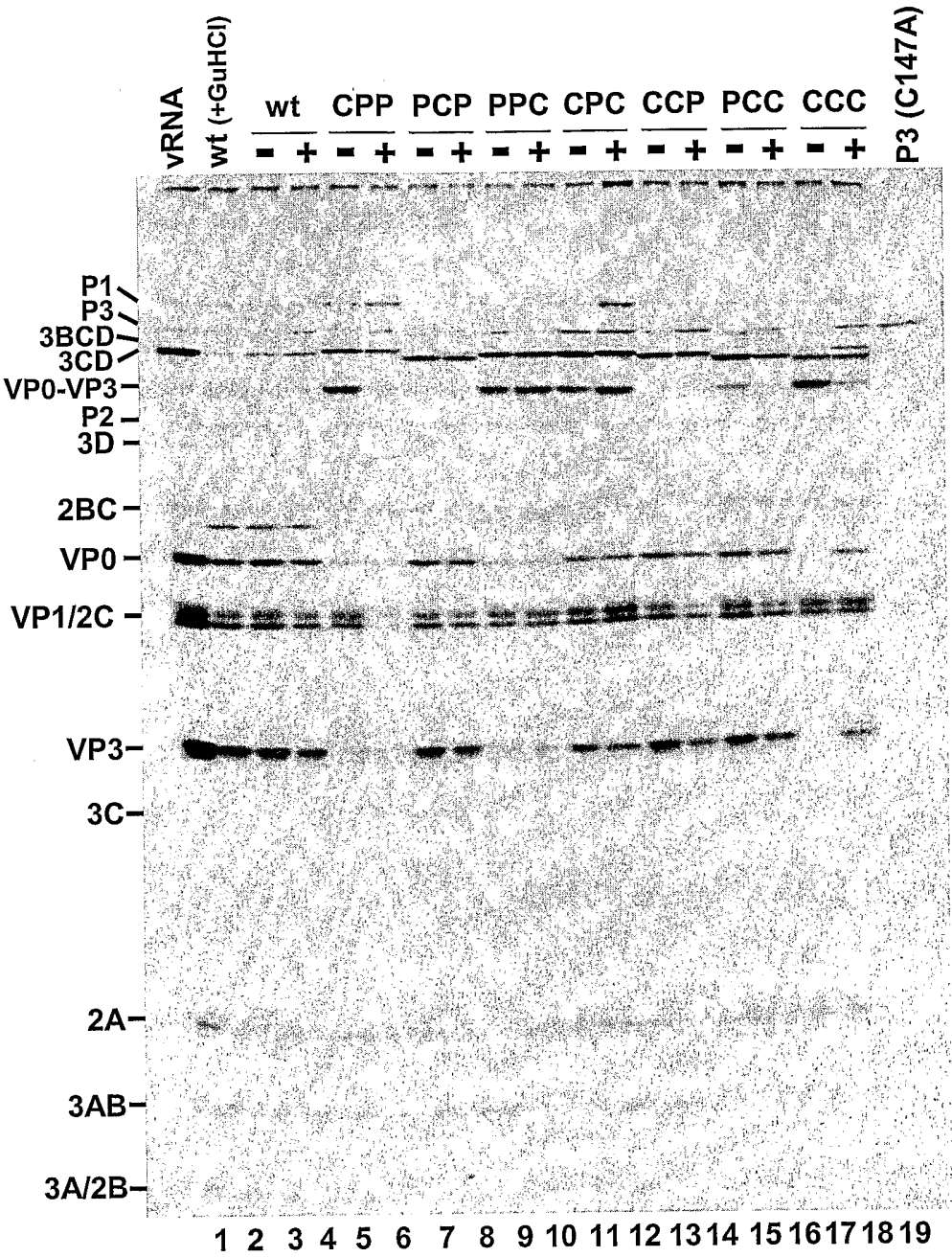


Figure 4B

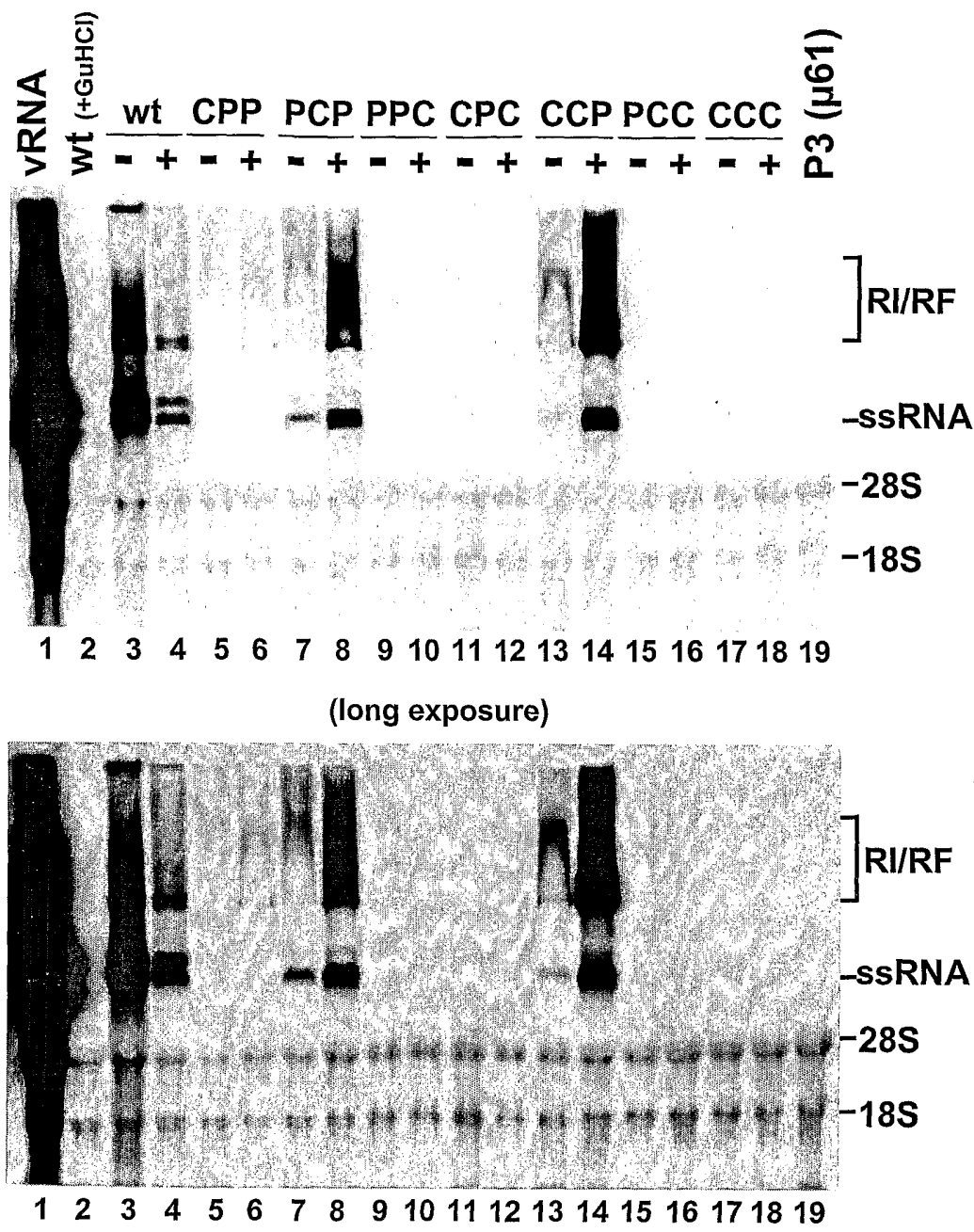


Figure 5A

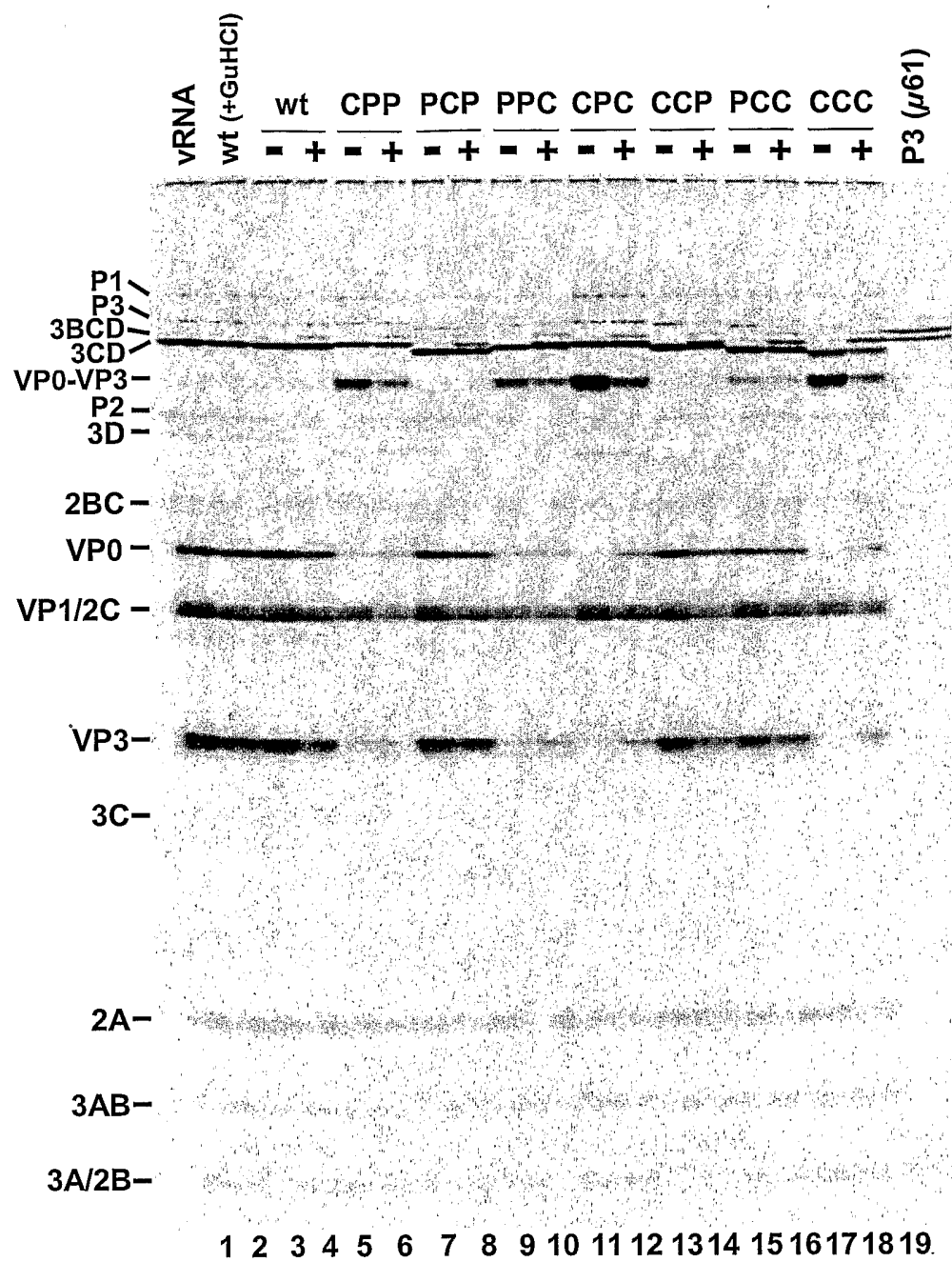


Figure 5B

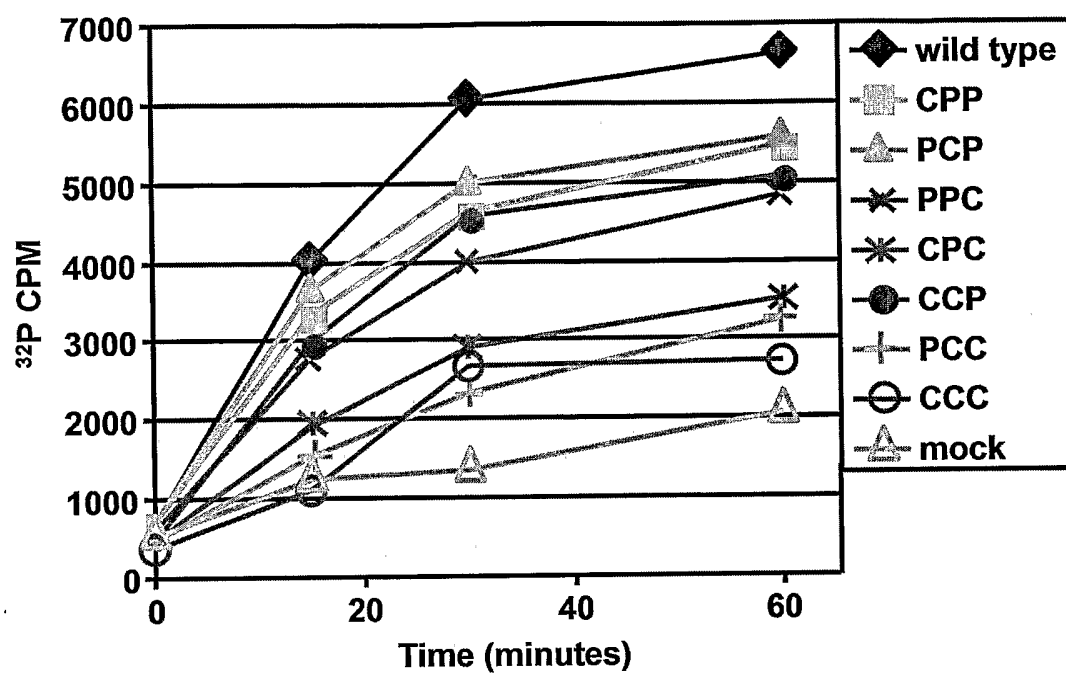


Figure 6

Sequence composition of PV1/CVB3 3D RNA polymerase

<u>RNA replication parameter</u>									
	CPP	PCP	PPC	CPC	CCP	PCC	CCC		
<i>Autonomous RNA replication</i>	<u>low</u> none	<u>low</u> low	<u>none</u> none	<u>none</u> none	<u>low</u> low	<u>none</u> none	<u>none</u> none		
<i>Complementation by wild type P3</i>	<u>high</u> <u>high</u>	<u>high</u> <u>high</u>	<u>low</u> <u>low</u>	<u>low</u> <u>low</u>	<u>high</u> <u>high</u>	<u>low</u> <u>low</u>	<u>low</u> <u>low</u>		
<i>Complementation by P3 defective in cloverleaf binding</i>	<u>high</u> <u>high</u>	<u>high</u> <u>high</u>	<u>low</u> <u>low</u>	<u>low</u> <u>low</u>	<u>high</u> <u>high</u>	<u>none</u> <u>none</u>	<u>none</u> <u>none</u>		
<i>Complementation by P3 defective in protein processing</i>	<u>high</u> <u>high</u>	<u>none</u> <u>low</u>	<u>low</u> <u>low</u>	<u>low</u> <u>low</u>	<u>high</u> <u>high</u>	<u>none</u> <u>none</u>	<u>none</u> <u>none</u>		
<i>Complementation by P3 with defective 3D polymerase</i>	<u>low</u> none	<u>high</u> <u>high</u>	<u>none</u> none	<u>none</u> none	<u>high</u> <u>high</u>	<u>none</u> <u>none</u>	<u>none</u> <u>none</u>		

(Negative-strand RNA synthesis)
Positive-strand RNA synthesis

Figure 7