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(54)发明名称

含人丙型肝炎病毒全长基因组的核酸构建物、核酸构建物转入其中的重组全长病毒基因组复制型细胞和生产丙型肝炎病毒颗粒的方法

(57)摘要

本发明提供有效复制含全长HCV基因组序列的RNA的方法和使用细胞培养系统生产含全长HCV复制子RNA或全长HCV基因组RNA的HCV病毒颗粒的方法。此外,本发明涉及生产丙型肝炎病毒颗粒的方法,其包括培养其中导入复制子RNA的细胞,并在培养基中产生病毒颗粒,其中所述复制子RNA包含的核苷酸序列含有基因型2a丙型肝炎病毒的全长基因组RNA序列、至少一种选择标记基因和/或至少一种报告基因以及至少一种IRES序列,或含有基因型2a丙型肝炎病毒的全长基因组RNA。再进一步,本发明还涉及丙型肝炎疫苗和抗丙型肝炎病毒颗粒的抗体。

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1. 一种复制子 RNA，所述 RNA 含有的核苷酸序列包含基因型 2a 丙型肝炎病毒基因组 RNA 的由 SEQ ID NO：1 组成的 5′ 非翻译区、由 SEQ ID NO：2 组成的核心蛋白编码序列、由 SEQ ID NO：3 组成的 E1 蛋白编码序列、由 SEQ ID NO：4 组成的 E2 蛋白编码序列、由 SEQ IDNO：5 组成的 NS2 蛋白编码序列、由 SEQ ID NO：6 组成的 NS3 蛋白编码序列、由 SEQ ID NO：7 组成的 NS4A 蛋白编码序列、由 SEQ ID NO：8 组成的 NS4B 蛋白编码序列、由 SEQ ID NO：9 组成的 NS5A 蛋白编码序列、由 SEQ ID NO：10 组成的 NS5B 蛋白编码序列和由 SEQ ID NO：11 组成的 3′ 非翻译区，至少一种选择标记基因和 / 或至少一种报告基因以及至少一种 IRES 序列，其中所述复制子 RNA 具有自主复制能力和感染性病毒颗粒生产能力。

2. 权利要求 1 的复制子 RNA，其中所述核苷酸序列以 5′ -3′ 方向按以下顺序包含 5′ 非翻译区、至少一种选择标记基因和 / 或至少一种报告基因、至少一种 IRES 序列，以及核心蛋白编码序列、E1 蛋白编码序列、E2 蛋白编码序列、NS2 蛋白编码序列、NS3 蛋白编码序列、NS4A 蛋白编码序列、NS4B 蛋白编码序列、NS5A 蛋白编码序列、NS5B 蛋白编码序列和 3′ 非翻译区。

3. 权利要求 1 的复制子 RNA，其中基因型 2a 丙型肝炎病毒基因组 RNA 是包含 SEQ ID NO：12 所示核苷酸序列的 RNA。

4. 一种复制子 RNA，所述 RNA 包含以下的 RNA：

(a) 含 SEQ ID NO：13 所示核苷酸序列的 RNA。

5. 一种生产细胞的方法，其中所述细胞复制复制子 RNA 并生产感染性病毒颗粒，所述方法包括将权利要求 1-4 中任一项的复制子 RNA 导入到细胞中。

6. 权利要求 5 的方法，其中所述细胞为增殖性细胞。

7. 权利要求 5 的方法，其中所述细胞为真核细胞。

8. 权利要求 7 的方法，其中所述真核细胞为人肝源细胞、人子宫颈源细胞或人胎肾源细胞。

9. 权利要求 7 的方法，其中所述真核细胞为 Huh7 细胞、HepG2 细胞、IMY-N9 细胞、HeLa 细胞或 293 细胞。

10. 权利要求 5 的方法获得的细胞，所述细胞复制复制子 RNA 并生产感染性病毒颗粒。

11. 一种生产丙型肝炎病毒颗粒的方法，所述方法包括培养权利要求 10 的细胞，以使该细胞生产感染性病毒颗粒。

12. 一种生产丙型肝炎病毒感染细胞的方法，所述方法包括培养权利要求 10 的细胞，并用培养物中的感染性病毒颗粒感染其它细胞。

13. 一种丙型肝炎病毒感染细胞，所述细胞利用权利要求 12 的方法获得。

14. 一种筛选抗丙型肝炎病毒的物质的方法，所述方法包括在测试物存在下培养选自以下 (a)、(b) 和 (c) 中的至少一种：

(a) 权利要求 10 的细胞，

(b) 权利要求 13 的丙型肝炎病毒感染细胞，

(c) 权利要求 11 的方法获得的丙型肝炎病毒颗粒和丙型肝炎病毒允许细胞；

并检测产生的培养物中的复制子 RNA 或病毒颗粒。

15. 一种生产丙型肝炎疫苗的方法，所述方法使用权利要求 11 的方法获得的丙型肝炎病毒颗粒或其部分作为抗原。

16. 一种生产用于基因治疗的嗜肝病毒载体的方法，所述方法使用权利要求 1-4 中任一项的复制子 RNA。

17. 一种嗜肝病毒载体，所述载体利用权利要求 16 的方法获得。

18. 一种在细胞中复制和 / 或表达外源基因的体外方法，所述方法包括将外源基因编码 RNA 插入到权利要求 1-4 中任一项的复制子 RNA 中，并将其导入到所述细胞中。

19. 权利要求 18 的方法，其中所述细胞是增殖性细胞。

20. 一种生产细胞的方法，其中所述细胞复制 RNA 并生产感染性病毒颗粒，所述方法包括将含 SEQ ID NO :12 所示核苷酸序列的 RNA 导入到细胞中。

21. 权利要求 20 的方法，其中所述细胞是增殖性细胞。

22. 一种生产感染性丙型肝炎病毒颗粒的方法，所述方法包括将含 SEQ ID NO :12 所示核苷酸序列的 RNA 导入到细胞中，并培养细胞，以使该细胞生产病毒颗粒。

23. 一种生产含外源基因的病毒载体的方法，所述方法包括将外源基因编码 RNA 插入到含 SEQ ID NO :12 所示核苷酸序列的 RNA 中，将其导入到细胞中，并培养细胞，以使其生产病毒颗粒。

24. 权利要求 1-4 中任一项的复制子 RNA 在培养细胞中产生感染性丙型肝炎病毒颗粒中的用途。

25. 权利要求 11 的方法产生的丙型肝炎病毒颗粒在产生抗所述丙型肝炎病毒颗粒的抗体中的用途。

含人丙型肝炎病毒全长基因组的核酸构建物、核酸构建物转入其中的重组全长病毒基因组复制型细胞和生产丙型肝炎病毒颗粒的方法

技术领域

[0001] 本发明涉及含丙型肝炎病毒全长基因组的核酸构建物、生产丙型肝炎病毒颗粒的体外方法和所生产的丙型肝炎病毒颗粒的用途。

背景技术

[0002] 丙型肝炎病毒 (HCV) 属于黄病毒科家族，是一种具有单链正义 RNA 基因组的病毒，已知引起丙型肝炎。

[0003] HCV 通过持续感染引起慢性肝炎。目前，在世界范围内观察到的慢性肝炎的重要病因是持续 HCV 感染。实际上，大约 50％的持续感染个体发展成慢性肝炎。这些患者约有 20％在 10-20 年间由慢性肝炎转变成肝硬化，这些患者中有一些进一步发展成晚期致死性病症，例如肝癌。

[0004] 丙型肝炎目前主要通过使用干扰素－α 或干扰素－β 的疗法或使用干扰素－α 和利巴韦林（一种嘌呤－核苷衍生物）组合的疗法治疗。但是，即使在实施这些疗法时，在所有受治疗患者中也仅有约 60％观察到疗效。当观察到疗效后终止治疗时，一半以上的患者疾病复发。

[0005] 一个重要目标是开发有效对抗丙型肝炎的治疗剂或预防剂。在工业化国家丙型肝炎发病率高，最终引发严重后果，目前还没有可用的病因疗法。因此，需要开发 HCV-特异性化学疗法和疫苗疗法。开发抗 HCV 药物的目标可为抑制 HCV 复制或抑制 HCV 感染细胞。

[0006] 最近，已制备了 HCV 亚基因组 RNA 复制子系统，作为 HCV 衍生的自主复制 RNA（参见特许文献 1、2 和 3，非特许文献 1-4）。在 HCV 亚基因组 RNA 复制子系统中，制备其中 HCV 基因组结构基因被去除并被药物选择性标记基因取代的 HCV 复制子 RNA，并将其导入培养细胞中，由此使复制子 RNA 在细胞中自主复制。使用这些系统使得有可能分析 HCV 的复制机制。但是，这是一个其中仅可在 HCV 病毒增殖和复制过程中评价病毒 RNA 复制的实验系统，不能分析被感染细胞中 HCV 病毒颗粒形成和胞外释放或感染另一细胞的过程。

[0007] 此时，只能在使用动物如黑猩猩的实验系统中评价 HCV 病毒颗粒形成和胞外释放以及感染另一细胞的过程（非特许文献 5）。但是，使用活生物（例如动物）的实验系统很复杂，非常难分析。因此，为了分析 HCV 病毒颗粒形成和胞外释放以及感染另一细胞的过程，为了生产具有抑制该过程的作用机制的抗 HCV 药物，必须建立重现该过程的高度简单化的实验系统，即在细胞培养实验系统中的 HCV 病毒颗粒生产系统。

[0008] 此外，一旦可使用细胞培养系统稳定提供 HCV 病毒颗粒，就有可能使用分子生物学技术对病毒减毒或生产非感染性 HCV 病毒，这可用于疫苗。

[0009] 特许文献 1：日本特许公开（公报）第 2001-17187 号

[0010] 特许文献 2：国际专利申请 PCT/JP03/15038

[0011] 特许文献 3：JP 特许申请第 2003-329082 号

[0012] 非特许文献 1 :Lohmann 等, Science, (1999)285,110-113 页

[0013] 非特许文献 2 :Blight 等, Science, (2000)290,1972-1974 页

[0014] 非特许文献 3 :Friebe 等, J.Virol., (2001)75(24) :12047-12057 页

[0015] 非特许文献 4 :Ikeda 等, J.Virol., (2002)76(6) :2997-3006 页

[0016] 非特许文献 5 :Kolykhalov 等, Science, (1997)277,570-574 页

[0017] 非特许文献 6 :Kato 等, Gastroenterology, (2003)125,1808-1817 页

[0018] 非特许文献 7 :Yanagi 等, Proc.Natl.Acad.Sci., (1997)96(16) :8738-8743 页

[0019] 非特许文献 8 :Okamoto 等, J.Gen.Virol., (1991)73,2697-26704 页

[0020] 非特许文献 9 :Aoyagi 等, J.Clin.Microbiol., (1999)37(6) :1802-1808 页

[0021] 发明公开

[0022] 本发明的目标是提供含全长 HCV 基因组序列的 RNA 的有效复制方法和在细胞培养系统中生产含全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 的 HCV 病毒颗粒的方法。本发明的目标迄今为止还从未实现过。

[0023] 为实现以上目标,本发明的发明人深入研究,结果开发出一种在细胞培养系统中生产 HCV 病毒颗粒的方法。即,本发明如下所述。

[0024] 一种复制子 RNA,其包含的核苷酸序列含有基因型 2a 丙型肝炎病毒基因组 RNA 的 5′ 非翻译区、核心蛋白编码序列、E1 蛋白编码序列、E2 蛋白编码序列、NS2 蛋白编码序列、NS3 蛋白编码序列、NS4A 蛋白编码序列、NS4B 蛋白编码序列、NS5A 蛋白编码序列、NS5B 蛋白编码序列和 3′ 非翻译区,至少一种选择标记基因和 / 或至少一种报告基因,以及至少一种 IRES 序列。

[0025] 在该复制子 RNA 中,优选核苷酸序列含在 5′ -3′ 方向为以下顺序的 5′ 非翻译区、至少一种选择标记基因和 / 或至少一种报告基因,和至少一种 IRES 序列,以及核心蛋白编码序列、E1 蛋白编码序列、E2 蛋白编码序列、NS2 蛋白编码序列、NS3 蛋白编码序列、NS4A 蛋白编码序列、NS4B 蛋白编码序列、NS5A 蛋白编码序列、NS5B 蛋白编码序列和 3′ 非翻译区。

[0026] 在此复制子 RNA 的更优选实施方案中,基因型 2a 丙型肝炎病毒基因组 RNA 是含 SEQ ID NO :12 所示核苷酸序列的 RNA。

[0027] 在此复制子 RNA 的再更优选实施方案中,5′ 非翻译区含 SEQ IDNO :1 所示核苷酸序列,核心蛋白编码序列含 SEQ ID NO :2 所示核苷酸序列,E1 蛋白编码序列含 SEQ ID NO :3 所示核苷酸序列,E2 蛋白编码序列含 SEQ ID NO :4 所示核苷酸序列,NS2 蛋白编码序列含 SEQ ID NO :5 所示核苷酸序列,NS3 蛋白编码序列含 SEQ ID NO :6 所示核苷酸序列,NS4A 蛋白编码序列含 SEQ ID NO :7 所示核苷酸序列,NS4B 蛋白编码序列含 SEQ ID NO :8 所示核苷酸序列,NS5A 蛋白编码序列含 SEQ ID NO :9 所示核苷酸序列,NS5B 蛋白编码序列含 SEQ ID NO :10 所示核苷酸序列,而 3′ 非翻译区含 SEQ ID NO :11 所示核苷酸序列。

[0028] 含以下 (a) 或 (b)RNA 的复制子 RNA :

[0029] (a) 含 SEQ ID NO :13 所示核苷酸序列的 RNA ;或

[0030] (b) 含通过缺失、置换或添加 1-100 个核苷酸而衍生自 SEQ ID NO :13 所示核苷酸序列的核苷酸序列、并具有自主复制能力和病毒颗粒生产能力的 RNA。

[0031] 一种生产细胞的方法,其中所述细胞复制复制子 RNA 并生产病毒颗粒,该方法包

括将上述 [1] 或 [2] 中任一项的复制子 RNA 导入到细胞中。

[0032] 用于该方法的细胞优选为增殖性细胞。同样或否则，用于该方法的细胞优选为真核细胞。

[0033] 用于该方法的真核细胞优选为人肝源细胞、人子宫颈源细胞或人胎肾源细胞。所述真核细胞更优选为 Huh7 细胞、HepG2 细胞、IMY-N9 细胞、HeLa 细胞或 293 细胞。

[0034] 利用上述 [3] 的方法可获得的细胞，其复制复制子 RNA 并生产病毒颗粒。

[0035] 生产丙型肝炎病毒颗粒的方法，包括培养上述 [4] 的细胞，以使该细胞生产病毒颗粒。

[0036] 利用上述 [5] 的方法可获得的丙型肝炎病毒颗粒。

[0037] 生产丙型肝炎病毒感染细胞的方法，包括培养上述 [4] 的细胞，并用培养物中的病毒颗粒感染其它细胞。

[0038] 通过上述 [7] 的方法可获得的丙型肝炎病毒感染细胞。

[0039] 筛选抗丙型肝炎病毒物质的方法，包括在测试物质存在下培养选自以下 (a)、(b) 和 (c) 中的至少一种：

[0040] (a) 上述 [4] 的细胞，

[0041] (b) 上述 [8] 的丙型肝炎病毒感染细胞，和

[0042] (c) 上述 [6] 的丙型肝炎病毒颗粒和丙型肝炎病毒允许细胞；

[0043] 并检测所获培养物中的复制子 RNA 或病毒颗粒。

[0044] 丙型肝炎疫苗，包含上述 [6] 的丙型肝炎病毒颗粒或其部分。

[0045] 使用上述 [6] 的丙型肝炎病毒颗粒或其部分作为抗原生产丙型肝炎疫苗的方法。

[0046] 通过使用上述 [1] 或 [2] 的复制子 RNA 生产用于基因治疗的嗜肝病毒载体的方法。

[0047] 利用上述 [12] 的方法可获得的嗜肝病毒载体。

[0048] 在细胞中复制和 / 或表达外源基因的方法，包括将编码外源基因的 RNA 插入到上述 [1]] 或 [2] 中任一项的复制子 RNA 中，并将其导入到所述细胞中。

[0049] 一种细胞生产方法，其中所述细胞复制 RNA 并生产病毒颗粒，该方法包括将含 SEQ ID NO：12 所示核苷酸序列的 RNA 导入到该细胞中。

[0050] 生产丙型肝炎病毒颗粒的方法，包括将含 SEQ ID NO：12 所示核苷酸序列的 RNA 导入到细胞中，并培养细胞，使该细胞生产病毒颗粒。

[0051] 上述 [15] 或 [16] 的方法，其中所述细胞为增殖性细胞。

[0052] 生产含外源基因的病毒载体的方法，包括将编码外源基因的 RNA 插入到含 SEQ ID NO：12 所示核苷酸序列的 RNA 中，将其导入到细胞中，并培养细胞，使该细胞生产病毒颗粒。

[0053] 抗上述 [6] 的丙型肝炎病毒颗粒的抗体。

[0054] 在本申请要求其优先权的日本特许申请第 2004-045489 号的说明书和附图中的内容结合到本文中。

[0055] 附图简述

[0056] 图 1 是一幅示意图，显示了构建模板 DNA 的方法，该模板 DNA 用于制备本发明的全长 HCV 复制子 HCV 或全长 HCV 基因组 RNA。图 1 的上部显示了质粒克隆 pJFH1 的结构，其通过将全长 HCV 基因组插入到 T7 启动子下游产生。图 1 的下部显示了含全长 HCV 基因组

序列的质粒克隆 pFGREP-JFH1 的结构，其中含新霉素抗性基因和 EMCV IRES 的 DNA 片段插入到 pJFH1 的 T7 启动子和 5′ 非翻译区下游。该图中显示的术语如下。T7 :T7 RNA 启动子，5′ UTR :5′ 非翻译区，C :核心蛋白，E1、E2 :包膜蛋白。NS2、NS3、NS4A、NS4B、4A、4B :非结构蛋白。3′ UTR :3′ 非翻译区。AgeI、PmeI、XbaI :限制酶 AgeI、PmeI 和 XbaI 的限制性位点。GDD :对应于 NS5B 蛋白活性中心的氨基酸基序 GDD 的位点。neo :新霉素抗性基因。EMCVIRES :脑心肌炎病毒内部核糖体进入位点 ；

[0057] 图 2 是一幅照片，显示了 RNA 印迹分析的结果，其表明 rJFH-1 在其中已导入全长 HCV 基因组 RNA rJFH-1 的 Huh7 细胞中复制 ；

[0058] 图 3 显示了培养基中 HCV 核心蛋白定量的结果。空心圆代表其中已导入 rJFH1 的细胞，实心圆代表其中已导入 rJFH1/GND 的细胞 ；

[0059] 图 4 图示了 HCV 核心蛋白和全长 HCV 基因组 RNA 的量，以及通过蔗糖密度梯度分级分离已导入 rJFH-1 的 Huh7 细胞的培养上清液所收集的各个级分的比重。实心圆、空心圆和阴影圆分别代表 HCV 核心蛋白、全长 HCV 基因组 RNA 和比重。

[0060] 图 5 是一幅照片，显示了其中已转染入全长 HCV 复制子 RNArFGREP-JFH1 的 Huh7 细胞的集落形成 ；

[0061] 图 6 是一幅照片，显示了全长 HCV 复制子 RNA 在全长 HCV 复制子 RNA 复制型细胞克隆中的复制，其中所述克隆已通过将 rFGREP-JFH1 转染入 Huh7 细胞中建立 ；

[0062] 图 7 是一幅照片，显示了使用宿主细胞的基因组 DNA 作为模板和新霉素抗性基因特异性引物的 PCR 扩增的结果，用于证实新霉素抗性基因整合入基因组 DNA 中。M :DNA 大小标记物，P :阳性对照，N :Huh7 细胞 ；

[0063] 图 8 是一幅照片，显示了蛋白质印迹分析的结果，其表明核心蛋白在其中已导入全长 HCV 复制子 RNA rFGREP-JFH1 的 Huh7 细胞中表达 ；

[0064] 图 9 是一幅照片，显示了蛋白质印迹分析的结果，其表明 NS3 蛋白在其中已导入全长 HCV 复制子 RNA rFGREP-JFH1 的 Huh7 细胞中表达 ；

[0065] 图 10 是一幅照片，显示了蛋白质印迹分析的结果，其表明 NS5A 蛋白在其中已导入全长 HCV 复制子 RNA rFGREP-JFH1 的 Huh7 细胞中表达 ；

[0066] 图 11 图示了 HCV 核心蛋白和全长 HCV 复制子 RNA 的量，以及通过蔗糖密度梯度分级分离已导入 rFGREP-JFH1 的 Huh7 细胞的培养上清液所收集的各个级分的比重。实心圆、空心圆和阴影圆分别代表 HCV 核心蛋白、全长 HCV 复制子 RNA 和比重 ；

[0067] 图 12 是一幅照片，显示了 Huh7 细胞的集落形成，其中已加入全长 HCV 复制子 RNA 复制型细胞培养上清液中的病毒颗粒。

[0068] 实施本发明的最佳方式

[0069] 本发明如下详细阐述。

[0070] 1. 全长 HCV 复制子 RNA

[0071] 丙型肝炎病毒 (HCV) 基因组是单股正链 RNA，含约 9600 个核苷酸。此基因组 RNA 含 5′ 非翻译区 (也称为 5′ NTR 或 5′ UTR)、由结构区和非结构区组成的翻译区以及 3′ 非翻译区 (也称为 3′ NTR 或 3′ UTR)。HCV 结构蛋白在结构区中编码，多种非结构蛋白在非结构区中编码。

[0072] 这样的 HCV 结构蛋白 (core、E1 蛋白和 E2 蛋白) 和非结构蛋白 (NS2、NS3、NS4A、

NS4B、NS5A 和 NS5B) 如下产生：首先将翻译区翻译成单个连续的多蛋白，然后通过蛋白酶限制性切割多蛋白释放。在这些结构蛋白和非结构蛋白（即 HCV 的病毒蛋白）中，core 是核心蛋白，E1 和 E2 是包膜蛋白。非结构蛋白是参与病毒自身复制的蛋白，已知 NS2 具有金属蛋白酶活性，已知 NS3 具有丝氨酸蛋白酶活性（在 N 端侧的 1/3) 和解旋酶活性（在 C 端侧的 2/3)。此外，NS4A 是 NS3 蛋白酶活性的辅因子，已报道 NS5B 具有 RNA 依赖性 RNA 聚合酶活性。

[0073] 本发明的发明人使用 HVC 基因组 RNA 构建了具有自主复制能力和病毒颗粒生产能力的复制子 RNA。

[0074] 已通过修饰 HCV 基因组 RNA 产生的具有自主复制能力的 RNA 在本文中叫做“复制子 RNA”或“RNA 复制子”。在本说明书中，源自 HCV 的复制子 RNA 还可叫做 HCV-RNA 复制子。含全长 HCV 基因组 RNA 的本发明复制子 RNA 在本文叫做“全长 HCV 复制子 RNA”。本发明的全长 HCV 复制子 RNA 具有生产病毒颗粒的能力。

[0075] 在本发明的全长 HCV 复制子 RNA 的优选实施方案中，丙型肝炎病毒优选为但不限于基因型 2a 丙型肝炎病毒。在本发明中，“基因型 2a 丙型肝炎病毒”或“基因型 2a HCV”指按照 Simmonds 等（参见 Simmonds, P. 等，Hepatology,(1994)10,1321-1324 页）的国际分类法鉴别为基因型 2a 的肝炎病毒。在本发明中，“基因型 2a 丙型肝炎病毒”或“基因型 2a HCV”不仅包括具有天然 HCV 基因组 RNA 的病毒，而且包括具有其中天然 HCV 基因组序列已被人工修饰的基因组 RNA 的病毒。基因型 2a HCV 的具体实例包括 JFH-1 毒株（参见日本特许公开（公告）第 2002-171978 号）。

[0076] 在本说明书中，“丙型肝炎病毒基因组 RNA”指含丙型肝炎病毒单链正义 RNA 基因组整个区域的核苷酸序列的 RNA。基因型 2a 丙型肝炎病毒的基因组 RNA 优选为但不限于含 SEQ ID NO：12 所示核苷酸序列的 RNA。

[0077] 本发明全长 HCV 复制子的一个实施方案是包含核苷酸序列的复制子 RNA，该核苷酸序列包含 5′ 非翻译区、核心蛋白编码序列、E1 蛋白编码序列、E2 蛋白编码序列、NS2 蛋白编码序列、NS3 蛋白编码序列、NS4A 蛋白编码序列、NS4B 蛋白编码序列、NS5A 蛋白编码序列、NS5B 蛋白编码序列和 3′ 非翻译区、至少一种选择标记基因或报告基因以及至少一种 IRES 序列。

[0078] 本发明的全长 HCV 复制子 RNA 不限于但优选包含：在 5′ -3′ 方向为以下顺序的 5′ 非翻译区、至少一种选择标记基因或报告基因、至少一种 IRES 序列、核心蛋白编码序列、E1 蛋白编码序列、E2 蛋白编码序列、NS2 蛋白编码序列、NS3 蛋白编码序列、NS4A 蛋白编码序列、NS4B 蛋白编码序列、NS5A 蛋白编码序列、NS5B 蛋白编码序列和 3′ 非翻译区。

[0079] 在本申请的说明书中，“5′ 非翻译区”(5′ NTR 或 5′ UTR)、“核心蛋白编码序列”（核心区或 C 区）、“E1 蛋白编码序列”(E1 区）、“E2 蛋白编码序列”(E2 区）、“NS2 蛋白编码序列”(NS2 区）、“NS3 蛋白编码序列”(NS3 区）、“NS4A 蛋白编码序列”(NS4A 区）、“NS4B 蛋白编码序列”(NS4B 区）、“NS5A 蛋白编码序列”(NS5A 区）、“NS5B 蛋白编码序列”(NS5B 区）和“3′ 非翻译区”(3′ NTR 或 3′ UTR)，以及其它特定区域或位点，以包含基因型 2a HCV 病毒 JFH-1 毒株（日本特许公开（公报）第 2002-171978 号）基因组完整区域的全长基因组 RNA(SEQ ID NO：12) 为基准定义。

[0080] 另外，本发明丙型肝炎病毒 (HCV) 基因组中的部分区域或位点可以 SEQ ID NO：

1-11 所示序列为基准定义，SEQ ID NO :1-11 是 JFH-1 毒株基因组 RNA(SEQ ID NO :12) 的部分核苷酸序列。JFH-1 毒株全长基因组 RNA(源自 JFH-1 克隆 ;SEQ ID NO :12) 的“5′ 非翻译区”包含 SEQ ID NO :1 所示核苷酸序列。“核心蛋白编码序列”包含 SEQ IDNO :2 所示核苷酸序列。“E1 蛋白编码序列”包含 SEQ ID NO :3 所示核苷酸序列。“E2 蛋白编码序列”包含 SEQ ID NO :4 所示核苷酸序列。“NS2 蛋白编码序列”包含 SEQ ID NO :5 所示核苷酸序列。“NS3 蛋白编码序列”包含 SEQ ID NO :6 所示核苷酸序列。“NS4A 蛋白编码序列”包含 SEQ ID NO :7 所示核苷酸序列。“NS4B 蛋白编码序列”包含 SEQ ID NO :8 所示核苷酸序列。“NS5A 蛋白编码序列”包含 SEQ IDNO :9 所示核苷酸序列。“NS5B 蛋白编码序列”包含 SEQ ID NO :10 所示核苷酸序列。“3′ 非翻译区”包含 SEQ ID NO :11 所示核苷酸序列。

[0081] 例如，HCV 源 RNA 序列中的区域或位点可由 SEQ ID NO :1-12 的核苷酸序列中的核苷酸编号限定，核苷酸编号通过比对 RNA 序列和 SEQ ID NO :1-12 所示核苷酸序列来确定。在比对中，可存在空位、添加、缺失、置换等。

[0082] 在本发明的更优选实施方案中，包含在全长 HCV 复制子 RNA 中的 5′ 非翻译区、核心蛋白编码序列、E1 蛋白编码序列、E2 蛋白编码序列、NS2 蛋白编码序列、NS3 蛋白编码序列、NS4A 蛋白编码序列、NS4B 蛋白编码序列、NS5A 蛋白编码序列、NS5B 蛋白编码序列和 3′ 非翻译区优选分别包含 SEQ ID NO :1-11 所示核苷酸序列。

[0083] 本发明的全长 HCV 复制子 RNA 的优选实施方案为含 SEQ ID NO :1-11 所示核苷酸序列、至少一种标记基因和 / 或报告基因和至少一种 IRES 序列的复制子 RNA。

[0084] 本发明中的“选择标记基因”指赋予细胞选择能力的基因，使得只有表达该基因的细胞才可被选择。选择标记基因的一般性实例包括抗生素抗性基因。本发明中优选的选择标记基因实例包括新霉素抗性基因、胸苷激酶基因、卡那霉素抗性基因、抗硫胺素抗性基因、腺苷酰转移酶基因、Zeocin 抗性基因和嘌呤霉素抗性基因。优选新霉素抗性基因和胸苷激酶基因，更优选新霉素抗性基因。但是，本发明中的选择标记基因不限于这些基因。

[0085] 此外，在本发明中，“报告基因”指其编码基因产物可作为该基因表达指示剂的标记基因。报告基因的一般性实例包括催化光发射反应或显色反应的酶的结构基因。本发明中的报告基因的优选实例包括源自转座子 Tn9 的氯霉素乙酰转移酶基因、大肠杆菌源的 β - 葡糖醛酸酶基因或 β - 半乳糖苷酶基因、荧光素酶基因、绿色荧光蛋白基因、得自水母的水母发光蛋白基因和分泌型胎盘碱性磷酸酶 (SEAP) 基因。但是，本发明中的报告基因不限于这些基因。

[0086] 在全长复制子 RNA 中可包含以上选择标记基因和报告基因中的仅一种，或者可同时包含二者。一种或多种选择标记基因或报告基因可存在于一个全长 HCV 复制子 RNA 中。

[0087] 在本发明中，“IRES 序列”指内部核糖体进入位点，其允许核糖体通过结合 RNA 内部启动翻译。本发明中的 IRES 序列的优选实例包括但不限于 EMCV IRES(脑心肌炎病毒的内部核糖体进入位点)、FMDV IRES 和 HCV IRES。更优选 EMCV IRES 和 HCV IRES，EMCVIRES 是最优选的序列。

[0088] 本发明的全长 HCV 复制子 RNA 的再更优选实施方案是含 SEQID NO :13 所示核苷酸序列的 RNA。此外，含在 SEQ ID NO :13 所示核苷酸序列中通过缺失、置换或添加 1-100 个核苷酸 (优选 1-30 个，更优选 1-10 个，再更优选 1-6 个，最优选 1 至若干 (2-5) 个核苷酸) 而衍生自 SEQ ID NO :13 所示核苷酸序列的核苷酸序列、具有自主复制能力和病毒颗粒生产

能力的复制子 RNA 是全长 HCV 复制子 RNA 的优选实施方案，也包括在本发明范围中。

[0089] 本发明的全长 HCV 复制子 RNA 还可包含编码任选外源基因的 RNA，其中所述外源基因要在全长复制子 RNA 已导入其中的细胞中表达。编码外源基因的 RNA 还可连接在 5′ 非翻译区下游，或连接在选择标记基因或报告基因的上游或下游，或连接在 3′ 非翻译区上游。编码外源基因的 RNA 可插入在核心蛋白编码序列、E1 蛋白编码序列、E2 蛋白编码序列、NS2 蛋白编码序列、NS3 蛋白编码序列、NS4A 蛋白编码序列、NS4B 蛋白编码序列、NS5A 蛋白编码序列和 NS5B 蛋白编码序列之间的任意位点。

[0090] 当含外源基因编码 RNA 的全长 HCV 复制子 RNA 在已导入其的细胞中翻译时，其可表达外源基因编码的基因产物。因此，含外源基因编码 RNA 的全长 HCV 复制子 RNA 还可适用于在细胞中生产外源基因的基因产物。

[0091] 本发明的全长 HCV 复制子 RNA 可进一步包含核酶。核酶连接在选择标记基因和 / 或报告基因下游，使得可通过核酶的自切割活性将选择标记基因和 / 或报告基因由 IRES 序列、核心蛋白编码序列、E1 蛋白编码序列、E2 蛋白编码序列、NS2 蛋白编码序列、NS3 蛋白编码序列、NS4A 蛋白编码序列、NS4B 蛋白编码序列、NS5A 蛋白编码序列和 NS5B 蛋白编码序列以及 3′ 非翻译区上切下。

[0092] 在本发明的全长 HCV 复制子 RNA 中，上述选择标记基因和 / 或报告基因、病毒蛋白编码序列和外源基因、核酶等被连接在一起，使得它们由全长 HCV 复制子 RNA 以正确的读框翻译。在这些序列中，全长复制子 RNA 编码的蛋白优选通过蛋白酶切割位点等互相连接，使得蛋白被翻译或表达为多蛋白，接着由蛋白酶切割成各个蛋白。

[0093] 本发明还涉及编码本发明复制子 RNA 的 DNA 载体，优选表达载体。

[0094] 在本发明中，RNA 的“自主复制能力”指 RNA 在导入细胞中时能够自主生长。RNA 的自主复制能力可通过以下方法证实，但该方法不是限制性的。用目的 RNA 转染 Huh7 细胞并培养。由所获得的培养细胞提取 RNA，并使用能够特异性检测导入 RNA 的探针对 RNA 进行 RNA 印迹杂交。目的 RNA 检测证实自主复制。在本说明书实施例中有关集落形成能力实验、HCV 蛋白表达验证、复制子 RNA 检测等的描述中阐述了证实自主复制能力的具体方法的实例。

[0095] 此外，在本发明中，RNA 的“病毒颗粒生产能力”指当 RNA 导入到细胞（例如培养细胞，如 Huh7 细胞）中时在细胞中产生病毒颗粒。病毒颗粒生产能力可通过例如将使用 RNA 特异性引物的 RT-PCR 法用于检测导入该 RNA 的细胞的培养上清液来证实。其还可通过对培养上清液进行蔗糖密度梯度法以分离病毒颗粒和通过检测 HCV 蛋白来证实。在本说明书实施例中有关集落形成能力实验、HCV 蛋白表达验证、复制子 RNA 检测等的描述中阐述了具体方法的实例。

[0096] 2. 全长 HCV 复制子 RNA 的制备

[0097] 可使用本领域技术人员已知的基因工程技术制备本发明的全长 HCV 复制子 RNA。可使用例如但不限于 JFH-1 毒株作为基因型 2a 丙型肝炎病毒，利用以下的方法制备全长 HCV 复制子 RNA。

[0098] 首先，常规地再构建对应于 JFH-1 毒株基因组 RNA 完整区域 (SEQ ID NO :12 ；该序列在国际 DNA 数据库中在检索号 AB047639 下）的 DNA，并将其插入到 RNA 启动子下游，以便制备 DNA 克隆。本文使用的“对应于 RNA 的 DNA”指具有通过用 T（胸腺嘧啶）取代 U（尿嘧

啶）而来源于 RNA 核苷酸序列的核苷酸序列的 DNA。以上 RNA 启动子优选包含在质粒克隆中。优选 RNA 启动子的实例不限于但包括 T7 RNA 启动子、SP6 RNA 启动子和 SP3 RNA 启动子，特别优选 T7 RNA 启动子。

[0099] 接着，将选择标记基因和 / 或报告基因以及 IRES 序列编码 DNA 插入到上述 DNA 克隆中。优选将选择标记基因和 / 或报告基因插入到 5′ 非翻译区下游，将 IRES 序列插入到更下游。

[0100] 随后，使用如上制备的 DNA 克隆作为模板，使用 RNA 聚合酶合成 RNA。RNA 合成可通过标准方法由 5′ 非翻译区开始。当 DNA 克隆为质粒克隆时，可用使用限制酶由质粒克隆切下的 DNA 片段作为模板合成 RNA。另外，优选要合成的 RNA 的 3′ 端与病毒基因组 RNA 的 3′ 非翻译区的末端具有相同序列，未添加或缺失其它序列。由此合成的 RNA 是本发明的全长 HCV 复制子 RNA。

[0101] 3. HCV 颗粒的制备

[0102] 可通过将如上所述制备的全长 HCV 复制子 RNA 导入细胞中，获得可复制全长 HCV 复制子 RNA（优选持续复制（即具有复制子 RNA 复制能力））的重组细胞。在本说明书中，复制全长 HCV 复制子 RNA 的重组细胞被称为“全长 HCV 复制子 RNA 复制型细胞”。

[0103] 全长 HCV 复制子 RNA 复制型细胞可生产病毒颗粒。生产的病毒颗粒在由 HCV 病毒蛋白组成的外壳中含全长 HCV 复制子 RNA。因此，本发明的全长 HCV 复制子 RNA 复制型细胞生产的病毒颗粒是 HCV 颗粒。也就是说，在本发明中，可通过培养全长 HCV 复制子 RNA 复制型细胞，在细胞培养系统中制备 HCV 颗粒。优选地，可通过培养全长 HCV 复制子 RNA 复制型细胞，并收集在培养物（优选培养上清液）中产生的病毒颗粒，获得 HCV 颗粒。

[0104] 或者，可利用通过将全长 HCV 基因组 RNA 导入到细胞中获得的重组细胞生产 HCV 颗粒。全长 HCV 基因组 RNA 在其中导入本发明的全长 HCV 基因组 RNA（优选来源于 JFH-1 克隆的全长 HCV 基因组 RNA，更优选具有 SEQ ID NO :12 所示核苷酸序列的 RNA）的细胞中高效复制。在本说明书中，复制全长 HCV 基因组 RNA 的细胞称为“全长 HCV 基因组 RNA 复制型细胞”。全长 HCV 基因组 RNA 复制型细胞可生产病毒颗粒。全长 HCV 基因组 RNA 复制型细胞生产的病毒颗粒在由 HCV 病毒蛋白组成的壳中包含全长 HCV 基因组 RNA。因此，由其中导入本发明的全长 HCV 基因组 RNA 的细胞生产的病毒颗粒是 HCV 颗粒。不限于但优选地可通过培养其中导入来源于 JFH-1 克隆的全长 HCV 基因组 RNA（例如具有 SEQ ID NO :12 所示核苷酸序列的 RNA）的细胞，在细胞培养系统中制备 HCV 颗粒。例如，可通过培养其中导入全长 HCV 基因组 RNA（例如具有 SEQ IDNO :12 所示核苷酸序列的 RNA）的细胞，并收集在培养物（优选培养上清液）中产生的病毒颗粒，获得 HCV 颗粒。

[0105] 对于要导入上述全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 的细胞，可使用任何细胞，只要其可被传代培养。这样的细胞优选为真核细胞，更优选为人细胞，再更优选为人肝源细胞、人子宫颈源细胞或人胎肾源细胞。优选可使用增殖性细胞，包括癌细胞系、干细胞系等细胞，更优选使用 Huh7 细胞、HepG2 细胞、IMY-N9 细胞、HeLa 细胞和 293 细胞等。对于这些细胞，可使用市售的细胞，这些细胞可得自细胞保藏机构，或者也可使用由任意细胞（例如癌细胞或干细胞）建立的细胞系。

[0106] 可使用本领域技术人员已知的任何技术，实现将全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 导入到细胞中。这样的导入方法的实例包括电穿孔、粒子枪法、脂转染法、磷酸

钙法、微注射法、DEAESepharose 法等。特别优选使用电穿孔的方法。

[0107] 全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 可单独导入，或者可在与其它核酸混合后导入。为改变全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 的量，同时将要导入的 RNA 量保持在一定水平，将需要量的要导入的全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 与由要导入 RNA 的细胞提取的总细胞 RNA 混合，以使总 RNA 量达到一定水平，然后混合物用于导入到细胞中。可按照使用的导入方法确定用于导入到细胞中的复制子 RNA 的量，其优选为 1pg-100μg，更优选为 10pg-10μg。

[0108] 可利用在全长 HCV 复制子 RNA 中的选择标记基因或报告基因的表达，选择全长 HCV 复制子 RNA 复制型细胞。具体地说，例如，可在培养基中培养这种进行了全长 HCV 复制子 RNA 细胞导入处理的细胞，其中细胞可由于表达选择标记基因而被选择。或者，在进行全长 HCV 复制子 RNA 细胞导入处理的细胞培养后，可检测报告基因（例如荧光蛋白）的表达。

[0109] 作为实例，当全长 HCV 复制子 RNA 包含新霉素抗性基因作为选择标记基因时，将用全长 HCV 复制子 RNA 进行电穿孔法的细胞接种入培养皿中。在培养 12-72 小时（优选 16-48 小时）后，以 0.05mg/ml-3.0mg/ml 的浓度将 G418（新霉素）加入到培养皿中。优选在接种后将细胞连续培养 10-40 天，更优选 14-28 天，同时一周更换培养基两次，可通过用结晶紫染色活细胞，选择复制导入的全长 HCV 复制子 RNA 的细胞的集落。

[0110] 可通过标准方法由形成的集落克隆细胞。由此获得的复制全长 HCV 复制子 RNA 的细胞克隆在本说明书中被称为“全长 HCV 复制子 RNA 复制型细胞克隆”。本发明的全长 HCV 复制子 RNA 复制型细胞包括全长 HCV 复制子 RNA 复制型细胞克隆。

[0111] 对于全长 HCV 复制子 RNA 复制型细胞，可通过检测复制的全长 HCV 复制子 RNA，确认全长 HCV 复制子 RNA 的选择标记基因或报告基因没有整合在宿主基因组 DNA 中，并进一步检测 HCV 蛋白，证实全长 HCV 复制子 RNA 在细胞或细胞克隆中的实际复制。

[0112] 可按照本领域技术人员已知的任意 RNA 检测方法，检测已复制的全长 HCV 复制子 RNA。例如，可通过使用全长 HCV 复制子 RNA 特异性 DNA 片段作为探针的 RNA 杂交法，检测由细胞提取的总 RNA 中的全长 HCV 复制子 RNA。

[0113] 而且，可通过例如但不限于对由细胞提取出的基因组 DNA 进行 PCR，以扩增至少一部分选择标记基因或报告基因，然后确认没有扩增产物，来证实全长 HCV 复制子 RNA 中的选择标记基因或报告基因没有整合在宿主基因组 DNA 中。因为一般认为在证实扩增产物的细胞中选择标记基因或报告基因可能已整合入宿主基因组中，所以有可能全长 HCV 复制子 RNA 自身不复制。在此情况下，可如下所述通过检测 HCV 蛋白进一步证实全长 HCV 复制子 RNA 的复制。

[0114] 可通过例如使抗 HCV 蛋白（其由导入的全长 HCV 复制子 RNA 表达）的抗体与提取的细胞蛋白反应，检测 HCV 蛋白。该方法可通过本领域技术人员已知的任意蛋白检测方法进行。具体地说，例如，可通过将由细胞提取出的蛋白样品印迹在硝酸纤维素膜上，使抗 HCV 蛋白抗体（例如抗 -NS3 特异性抗体或由丙型肝炎患者收集的抗血清）与硝酸纤维素膜反应，并检测抗 HCV 蛋白抗体，检测 HCV 蛋白。如果在提取的细胞蛋白中检测出 HCV 蛋白，则可推断该细胞复制全长 HCV 复制子 RNA，并表达 HCV 蛋白。

[0115] 可通过本领域技术人员已知的任意病毒检测方法，证实全长 HCV 复制子 RNA 复制型细胞或全长 HCV 基因组 RNA 复制型细胞的病毒颗粒生产能力。例如，通过蔗糖密度梯度

分级分离怀疑生产病毒颗粒的细胞的培养上清液，并测定各个级分的级分密度、HCV 核心蛋白浓度以及全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 的量。结果，如果核心蛋白的峰与全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 的峰一致，显示检测峰的级分的密度（例如 1.18-1.20mg）小于分级分离 25％ NP40（聚氧乙烯 (9) 辛基苯基醚）处理的培养上清液所获得的等同级分的密度，则可认为细胞具有病毒颗粒生产能力。

[0116] 例如可使用核心蛋白、E1 蛋白或 E2 蛋白的抗体检测培养上清液中释放的 HCV 病毒颗粒。另外，可通过使用特异性引物的 RT-PCR 法扩增和检测培养上清液中的全长 HCV 复制子 RNA，间接检测 HCV 病毒颗粒的存在。

[0117] 4. 用本发明的 HCV 颗粒感染另一细胞

[0118] 本发明的 HCV 病毒颗粒具有感染细胞（优选 HCV 允许细胞）的能力。本发明还涉及生产丙型肝炎病毒感染细胞的方法，包括培养全长 HCV 复制子 RNA 复制型细胞或全长 HCV 基因组 RNA 复制型细胞，并用在由此获得的培养物（优选培养上清液）中的病毒颗粒感染另一细胞（优选 HCV 允许细胞）。在本发明中，HCV 允许细胞指对 HCV 敏感的细胞，优选但不限于肝细胞或淋巴系细胞。具体地说，肝细胞包括原代肝细胞、Huh7 细胞、HepG2 细胞、IMY-N9 细胞、HeLa 细胞、293 细胞等。淋巴系细胞包括但不限于 Molt4 细胞、HPB-Ma 细胞、Daudi 细胞等。

[0119] 当用本发明的全长 HCV 复制子 RNA 复制型细胞生产的 HCV 颗粒感染细胞（例如 HCV 允许细胞）时，全长 HCV 复制子 RNA 在感染细胞中复制，病毒颗粒也在感染细胞中形成。因为用在全长 HCV 复制子 RNA 复制型细胞中产生的病毒颗粒感染的细胞表达选择标记基因和 / 或报告基因，所以可利用表达选择和 / 或检测感染细胞。通过用在本发明的全长 HCV 复制子 RNA 复制型细胞中产生的病毒颗粒感染细胞，全长 HCV 复制子 RNA 在细胞中复制，可进一步生产病毒颗粒。

[0120] 更进一步，通过用在本发明的全长 HCV 基因组 RNA 复制型细胞中产生的 HCV 颗粒感染细胞（例如 HCV 允许细胞），全长 HCV 基因组 RNA 在感染细胞中复制，病毒颗粒也在感染细胞中形成。通过用在本发明的全长 HCV 基因组 RNA 复制型细胞中产生的病毒颗粒感染细胞，全长 HCV 基因组 RNA 在细胞中复制，而且可生产病毒颗粒。

[0121] 在全长 HCV 复制子 RNA 复制型细胞或全长 HCV 基因组 RNA 复制型细胞中产生的 HCV 病毒颗粒可感染 HCV 允许动物，例如黑猩猩等，并在其中诱发由 HCV 引起的肝炎。

[0122] 5. 本发明的其它实施方案

[0123] 全长 HCV 复制子 RNA 在本发明的全长 HCV 复制子 RNA 复制型细胞中高效复制。另外，全长 HCV 基因组 RNA 在本发明的全长 HCV 基因组 RNA 复制型细胞中高效复制。因此，可使用本发明的全长 HCV 复制子 RNA 复制型细胞或全长 HCV 基因组 RNA 复制型细胞高效生产全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA。

[0124] 在本发明中，可通过培养全长 HCV 复制子 RNA 复制型细胞，由培养物（培养细胞和 / 或培养基）中提取 RNA，对 RNA 进行电泳法，并分离和纯化全长 HCV 复制子 RNA，生产全长 HCV 复制子 RNA。还可通过相似的方法，使用全长 HCV 基因组 RNA 复制型细胞生产全长 HCV 基因组 RNA。通过这样的方法生产的 RNA 包含丙型肝炎病毒的全长基因组序列。在此情况下，可通过选择标记基因和 / 或报告基因和 IRES 序列中断丙型肝炎病毒的全长基因组序列。借助于包含所提供的丙型肝炎病毒全长基因组序列的 RNA 的生产方法，使得有可能更

详细地分析丙型肝炎病毒基因组。

[0125] 再者，本发明的全长 HCV 复制子 RNA 复制型细胞或全长 HCV 基因组 RNA 复制型细胞可适用于生产 HCV 蛋白。HCV 蛋白可利用本领域技术人员已知的任何方法生产。例如，可通过将全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 导入到细胞中，培养重组细胞，并利用已知方法由如此获得的培养物（培养细胞和 / 或培养基）中收集蛋白，生产 HCV 蛋白。

[0126] 再者，本发明的 HCV 病毒颗粒可具有嗜肝性。因此，可使用本发明的全长 HCV 复制子 RNA 生产嗜肝病毒载体。该病毒载体适用于基因治疗。在本发明中，通过将外源基因编码 RNA 整合入全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 中并将整合的 RNA 导入细胞中表达，外源基因可被导入到细胞中，并在细胞中复制。此外，通过制备其中全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 的 E1 蛋白编码序列和 / 或 E2 蛋白编码序列被其它生物物种来源病毒的外壳蛋白编码序列置换的 RNA，使该 RNA 有可能感染不同的生物物种。还是在此情况下，外源基因整合入全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 中，这可用作在肝细胞中表达外源基因的嗜肝病毒载体。

[0127] 本发明还涉及生产携带外源基因的病毒载体的方法，包括将外源基因编码 RNA 插入到含 SEQ ID NO :12 所示核苷酸序列的 RNA 中，将其导入到细胞中，并培养细胞，以生产病毒颗粒。

[0128] 本发明提供含本发明的 HCV 颗粒或其部分的丙型肝炎疫苗，生产含本发明的 HCV 颗粒或其部分的丙型肝炎疫苗的方法。

[0129] 具体地说，如上制备的 HCV 颗粒可直接用作疫苗，或者可在利用本领域已知方法减毒或灭活后使用。例如，可通过使用柱层析、过滤、离心等纯化 HCV 颗粒，获得 HCV 疫苗母液。可由此 HCV 疫苗母液制备减毒活 HCV 疫苗或灭活 HCV 疫苗。可通过例如将灭活剂（如福尔马林、β - 丙内酯、戊二醛等）加入到病毒悬浮液中并混合，使其与病毒反应 (Appaiahgari 等，Vaccine，(2004)22(27-28)，3669-3675 页），进行病毒灭活。

[0130] 为生产本发明的疫苗，有可能使用其中通过使用公开已知的技术引入突变减弱或消除病原性的 HCV 复制子 RNA。

[0131] 本发明的疫苗制备成溶液或悬浮液给予。还有可能制备为适于溶解或悬浮在液体中的固体物质形式。制品可在脂质体中乳化或囊化。活性免疫原性组分如 HCV 颗粒经常与药物可接受的并适于活性成分的赋形剂混合。合适的赋形剂包括例如水、生理盐水、葡萄糖、甘油、乙醇及其混合物。此外，如果有需要，疫苗可包含少量的助剂（例如增湿剂或乳化剂）、pH 缓冲剂和 / 或用于增强疫苗效力的佐剂。有效佐剂的实例包括但不限于以下物质 :氢氧化铝、N- 乙酰 - 胞壁酰 -L- 苏氨酰 -D- 异谷氨酰胺 (thr-MDP)、N- 乙酰 - 正 - 胞壁酰 -L- 丙氨酰 -D- 异谷氨酰胺 (CGP11637，nor-MDP)、N- 乙酰胞壁酰 -L- 丙氨酰 -D- 异谷氨酰胺 -L- 丙氨酸 -2-(1′ -2′ - 二棕榈酰 -sn- 甘油 -3- 羟基磷酰氧基）- 乙胺 (CGP19835A，称为 MTP-PE) 和 RIBI。RIBI 在 2％鲨烯 /Tween ® 80 乳浊液中包含三种提取自细菌的组分，即单磷酰脂质 A、二分枝菌酸海藻糖和细胞壁骨架 (HPL+TDM+CWS)。可通过检测抗免疫原性 HCV 颗粒的抗体的量测定佐剂效力，其中所述抗体通过给予由 HCV 颗粒组成的疫苗产生。

[0132] 本发明的疫苗一般胃肠外给予，例如通过注射，如皮下或肌内注射。适于其它给予途径的其它剂型包括栓剂，在某些情况下包括口服制剂。

[0133] 如果有需要，可将一种或多种具有佐剂活性的上述化合物加入到 HCV 疫苗中。佐剂为此免疫系统的非特异性刺激因子，在宿主中增强对 HCV 疫苗的免疫应答。该技术领域已知佐剂的具体实例包括完全弗氏佐剂、不完全弗氏佐剂、维生素 E、非离子型嵌段共聚物、胞壁酰二肽、皂苷、矿物油、植物油和 Carbopol。特别适用于粘膜的佐剂包括例如大肠杆菌不耐热毒素 (LT) 和霍乱毒素 (CT)。其它合适的佐剂包括例如氢氧化铝、磷酸铝或氧化铝、油乳浊液（例如 Bayol（注册商标）或 Marcol 52（注册商标））、皂苷或维生素 E 增溶物。在优选的实施方案中，本发明的疫苗包含佐剂。

[0134] 例如，对于皮下、皮内、肌内和静脉内给予的注射，本发明的 HCV 疫苗中可包含的药物可接受的载体和稀释剂的具体实例包括稳定剂、糖类（例如山梨醇、甘露醇、淀粉、蔗糖、葡萄糖、葡聚糖）、蛋白（例如白蛋白或酪蛋白）、含蛋白的物质（例如牛血清白蛋白或脱脂奶）和缓冲液（例如磷酸盐缓冲液）。

[0135] 用于栓剂的常规粘合剂和载体包括例如聚亚烷基二醇或甘油三酯。栓剂可由含 0.5％ -50％（优选 1％ -20％）活性成分的混合物配制。口服制剂可包含通常使用的赋形剂。这样的赋形剂包括例如药用级的甘露醇、乳糖、淀粉、硬脂酸镁、糖精钠、纤维素、碳酸镁等。

[0136] 本发明的疫苗可以溶液、悬浮液、片剂、丸剂、胶囊、延迟释放制剂或粉末剂型生产，并可包含 10-95％（优选 25-70％）的活性成分（病毒颗粒或其部分）。

[0137] 本发明的疫苗通过适于剂型的方法并以预防和 / 或治疗有效量给予。剂量为 0.01μg-100,000μg，这取决于要治疗的患者、患者免疫系统中的抗体形成能力和需要的保护水平。其还依赖于给予途径，例如口服、皮下、皮内、肌内、静脉内等。

[0138] 该疫苗可通过单次给予程序给予，或优选通过复合给予程序给予。在复合给予程序中，在给予开始时进行 1-10 次单独给予，接着以维持和 / 或增强免疫反应所需要的间隔给予。例如，可在 1-4 个月后进行另一类给予，作为第二次给予。必要时，可在几个月后持续给予。给予方案至少部分地按照个体患者的需要来确定，取决于主治医师的判断。

[0139] 此外，含免疫原性 HCV 颗粒的疫苗可与其它免疫控制剂（例如免疫球蛋白）共同给予。

[0140] HCV 颗粒疫苗可通过给予健康个体，以诱发对 HCV 的免疫应答，用于预防性地对抗可能的新 HCV 感染。HCV 颗粒疫苗还可通过给予感染 HCV 的患者并在体内诱发针对 HCV 的强免疫应答，用作治疗性疫苗，以清除 HCV。

[0141] 全长 HCV 复制子 RNA 复制型细胞或全长 HCV 基因组 RNA 复制型细胞或用在这些细胞中产生的病毒颗粒感染的丙型肝炎病毒感染细胞，可用作测试系统，用于筛选促进或抑制例如丙型肝炎病毒复制、病毒颗粒重构和病毒颗粒释放的物质（抗丙型肝炎病毒的物质）。具体地说，例如，可通过确定测试物是否促进或抑制全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 的复制或者病毒颗粒的形成或释放，在测试物存在下培养这些细胞，并检测所获培养物中的全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 或病毒颗粒，筛选促进或抑制丙型肝炎病毒生长的物质。在此情况下，可通过测定由上述细胞提取的 RNA 制品中的全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 的量、比率或存在情况，对培养物中的全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 进行检测。可通过检测培养上清液中 HCV 蛋白的量、比率或存在情况，对培养物（主要是培养上清液）中的病毒颗粒进行检测。

[0142] 而且，可通过检测该培养物中的病毒颗粒，研究由 HCV 感染患者的血清纯化的免疫球蛋白是否可预防本发明 HCV 颗粒的感染。在此测试中，可使用已用本发明的 HCV 病毒颗粒免疫的小鼠、大鼠、兔等的血清。可使用采用部分 HCV 蛋白、HCV 基因等进行的免疫。可以相似的方式对其它感染预防性物质进行该测试。

[0143] 产生的抗本发明 HCV 病毒颗粒的本发明抗体包括多克隆抗体和单克隆抗体。当优选多克隆抗体时，第一步用本发明的 HCV 颗粒免疫选定的哺乳动物（例如小鼠、兔、山羊、绵羊、马等）。由被免疫动物收集血清，并用已知方法加工。如果含针对 HCV 表位的多克隆抗体的血清包含针对其它抗原的抗体，则可通过免疫亲和层析纯化这些血清。生产多克隆抗血清的方法及其治疗方法是本领域已知的。可由已感染 HCV 的哺乳动物分离多克隆抗体。

[0144] 本领域技术人员可容易地生产针对 HCV 表位的单克隆抗体。已知的常用方法是生产产生单克隆抗体的杂交瘤。例如，可使用在 Current Protocols in Immunology(John Wiley & Sons, Inc.) 中描述的方法。

[0145] 可通过细胞融合或其它方法，例如用肿瘤基因 DNA 直接转化 B 淋巴细胞或用 EB 病毒转导，生产产生单克隆抗体的细胞系。

[0146] 通过这些方法获得的单克隆抗体和多克隆抗体用于诊断、治疗和预防 HCV。

[0147] 使用本发明的 HCV 颗粒生产的抗体可与药物可接受的增溶剂、添加剂、稳定剂、缓冲剂等一起给予。可选择任何给予途径，但优选皮下、皮内和肌内给予，更优选静脉内给予。

[0148] 在本发明的全长 HCV 复制子 RNA 复制型细胞或全长 HCV 基因组 RNA 复制型细胞中产生的 HCV 颗粒和 HCV 允许细胞可作为测试系统，用于筛选可刺激或抑制 HCV 与细胞结合的物质。具体地说，例如，可通过在测试物存在下培养本发明的全长 HCV 复制子 RNA 复制型细胞中产生的 HCV 颗粒和 HCV 允许细胞，检测所获培养物中的全长 HCV 复制子 RNA 或病毒颗粒，并确定测试物是否促进或抑制复制子 RNA 复制或病毒颗粒形成，筛选可促进或抑制丙型肝炎病毒生长的物质。

[0149] 可按照上述技术或以下实施例对全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 进行这样的检测。上述测试系统可用于生产和评价丙型肝炎病毒感染的预防剂、治疗剂或诊断剂。

[0150] 具体地说，上述本发明测试系统的应用实例包括以下几点：

[0151] (1) 筛选抑制 HCV 生长和感染的物质

[0152] 抑制 HCV 生长和感染的物质包括例如直接或间接影响 HCV 生长和感染的有机化合物、通过与 HCV 基因组或其互补链中的靶序列杂交而直接或间接影响 HCV 生长或 HCV 蛋白翻译的反义寡核苷酸等。

[0153] (2) 评价在细胞培养物中具有抗病毒活性的各种物质

[0154] 前述各种物质包括通过合理药物设计或高通量筛选获得的物质（例如纯化和分离的酶）。

[0155] (3) 鉴别治疗 HCV 感染患者的新靶标

[0156] 例如，本发明的全长 HCV 复制子 RNA 复制型细胞或全长 HCV 基因组 RNA 复制型细胞可用于鉴别可在 HCV 生长中起重要作用的宿主细胞蛋白。

[0157] (4) 评价 HCV 对药物等的抗性获得能力，并鉴别与抗性相关的突变

[0158] (5) 生产病毒蛋白作为抗原，用于开发、生产和评价丙型肝炎病毒感染的诊断剂和

治疗剂

[0159] (6) 生产病毒蛋白作为抗原，用于开发、生产和评价用于丙型肝炎病毒感染的疫苗和生产减毒 HCV

[0160] (7) 生产用于诊断和治疗丙型肝炎病毒感染的单克隆或多克隆抗体。

[0161] 基于以下的实施例和附图将更具体地描述本发明。但本发明的技术范围不限于这些实施例。

[0162] 实施例 1 :得自全长 HCV 基因组 RNA 的全长 HCV 复制子 RNA 的制备

[0163] (A) 构建表达载体

[0164] 构建质粒 DNA，其中 DNA(JFH-1 克隆) 插入到 pUC19 质粒中 T7RNA 启动子序列的下游，此 DNA(JFH-1 克隆) 含已由暴发性肝衰竭患者中分离出的丙型肝炎病毒 JFH-1 毒株 (基因型 2a) 的全长基因组 cDNA。

[0165] 具体地说，将通过扩增 JFH-1 毒株的病毒 RNA 所获得的 RT-PCR 片段克隆入 pGEM-T EASY 载体 (Promega) 中，以获得质粒 pGEM1-258、pGEM44-486、pGEM317-849、pGEM617-1323、pGEM1141-2367、pGEM2285-3509、pGEM3471-4665、pGEM4547-5970、pGEM5883-7003、pGEM6950-8035、pGEM7984-8892、pGEM8680-9283、pGEM9231-9634 和 pGEM9594-9678(参见非特许文献 6)。通过使用 PCR 法和限制酶，将包含在这些质粒中的病毒基因组 RNA 来源的 cDNA 连接在一起，以克隆全长病毒基因组 cDNA。将 T7R RNA 启动子序列插入到全长病毒基因组 cDNA 的上游。在下文中，以此方式构建的质粒 DNA 称为 pJFH1(图 1 的上部)。上述 JFH-1 克隆的制备已描述于特许文献 1 和非特许文献 3。此外，JFH-1 克隆的全长 cDNA 的核苷酸序列以检索号 AB047639 登记在国际 DNA 数据库 (DDBJ/EMBL/GenBank) 中。

[0166] 接着，通过将 EMCV-IRES(脑心肌炎病毒的内部核糖体进入位点) 和新霉素抗性基因 (neo ；也称为新霉素磷酸转移酶基因) 插入到 pJFH1 质粒 DNA(图 1 的下部) 的 5′ 非翻译区和核心蛋白区之间，构建质粒 DNA pFGREP-JFH1。该构建方法与先前的公开内容 (非特许文献 4) 一致。此外，通过导入突变制备突变质粒克隆 pJFH1/GND 和 pFGREP-JFH1/GND，其中所述突变将对应于 RNA 聚合酶 (由 pJFH1 和 pFGREP-JFH1 中的 NS5B 区编码) 活性中心的氨基酸基序 GDD 变为 GND。因为由突变克隆 pJFH1/GND 和 pFGREP-JFH1/GND 编码的 NS5B 蛋白活性位点的氨基酸序列被改变，所以不能由突变克隆表达复制复制子 RNA 所需的活性 NS5B 蛋白。

[0167] 此外，通过将荧光素酶基因插入到 pFGREP-JFH1 的第 415-420 的 MluI 位点和第 2075-2082 的 PmeI 位点之间，以用荧光素酶基因替代 pFGREP-JFH1 的新霉素抗性基因，制备 pFGREP-JFH1/Luc，作为导入报告基因的表达载体。另外，通过将 pFGREP-JFH1/Luc 第 10933 位的 G 突变为 A，制备突变体 pFGREP-JFH1/Luc/GND，其中 NS5bRNA 聚合酶活性中心的 GDD 基序被变为 GND。

[0168] 通过将绿色荧光蛋白基因插入到 pFGREP-JFH1 的第 415-420 的 MluI 位点和第 1142-1149 的 PmeI 位点之间，制备 pFGREP-JFH1/EGFP，其中 pFGREP-JFH1 的新霉素抗性基因被绿色荧光蛋白基因取代。另外，通过将 pFGREP-JFH1/EGFP 第 10000 位的 G 突变为 A，制备突变体 pFGREP-JFH1/EGFP/GND，其中 NS5b RNA 聚合酶活性中心的 GDD 基序被变为 GND。

[0169] 通过将分泌型胎盘碱性磷酸酶基因插入到 pFGREP-JFH1 的第 415-420 的 MluI 位点和第 1982-1989 的 PmeI 位点之间，以用分泌型胎盘碱性磷酸酶基因取代 pFGREP-JFH1 的

新霉素抗性基因，制备 pFGREP-JFH1/SEAP。另外，通过将 pFGREP-JFH1/SEAP 第 10840 位的 G 突变为 A，制备突变体 pFGREP-JFH1/SEAP/GND，其中 NS5bRNA 聚合酶活性中心的 GDD 基序被变为 GND。

[0170] (B) 全长 HCV 基因组 RNA 和全长 HCV 复制子 RNA 的制备

[0171] 如上构建表达载体，用限制酶 XbaI 消化 pJFH1、pJFH1/GND、pFGREP-JFH1 和 pFGREP-JFH1/GND，以制备模板 DNA，用于合成全长 HCV 基因组 RNA 和全长 HCV 复制子 RNA。随后，通过于 30℃温育 30 分钟，用 20U 绿豆核酸酶的 50μl 反应溶液处理 10-20μg 各 XbaI 片段。绿豆核酸酶是这样一种酶：其催化涉及选择性消化双链 DNA 的单链部分的反应。通常，如果使用以上 XbaI 片段原样作为模板合成 RNA，则合成在 3′ 端具有 4 个额外碱基 CUGA 的复制子 RNA，其为 XbaI 识别位点的一部分。因此，在该实施例中，通过用绿豆核酸酶处理 XbaI 片段，由 XbaI 片段去除 4 碱基 CUGA。随后，对绿豆核酸酶处理后的含 XbaI 片段的溶液进行标准蛋白去除处理，以获得无 4 碱基 CUGA 的纯化 XbaI 片段，作为以下使用的模板 DNA。

[0172] 接着，使用 T7 RNA 聚合酶由该模板体外合成 RNA。使用 MEGAscript(Ambion Co.) 进行 RNA 合成。按照生产商的说明书使 20μl 含 0.5-1.0μg 模板 DNA 的反应混合物反应。

[0173] 在 RNA 合成后，将 DNA 酶 (2U) 加入到反应混合物中，于 37℃反应 15 分钟，然后用酸 - 苯酚处理提取 RNA，以去除模板 DNA。以此方式由源自 pJFH1、pJFH1/GND、pFGREP-JFH1 和 pFGREP-JFH1/GND 的模板 DNA 合成的 RNA 分别称为 rJFH1、rJFH1/GND、rFGREP-JFH1 和 rFGREP-JFH1/GND。这些 RNA 的核苷酸序列分别示于 SEQ ID NO：12、13、14 和 15 的 rJFH-1、rFGREP-JFH1、rJFH1/GND 和 rFGREP-JFH1/GND。rJFH1 是本发明全长 HCV 基因组 RNA 的实例，与 JFH-1 毒株的全长 HCV 基因组具有相同的序列结构。rFGREP-JFH1 是本发明全长 HCV 复制子 RNA 的实例。

[0174] 随后，通过分别使用如上制备的表达载体 pFGREP-JFH1/Luc、pFGREP-JFH1/Luc/GND、pFGREP-JFH1/EGFP、pFGREP-JFH1/EGFP/GND、pFGREP-JFH1/SEAP 和 pFGREP-JFH1/SEAP/GND 作为模板，生产为 HCV 复制子 RNA 的 rFGR-JFH1/Luc(SEQ ID NO：21)、rFGR-JFH1/Luc/GND((SEQ ID NO：22)、rFGR-JFH1/EGFP(SEQID NO：23)、rFGR-JFH1/EGFP/GND(SEQ ID NO：24)、rFGR-JFH1/SEAP(SEQ ID NO：25) 和 rFGR-JFH1/SEAP/GND(SEQ ID NO：26)。

[0175] 实施例 2：全长 HCV 基因组 RNA 在细胞中的复制和病毒颗粒的产生

[0176] (C) 全长 HCV 基因组 RNA 在细胞中的复制和病毒颗粒的产生

[0177] 将如上合成的各种量的全长 HCV 基因组 RNA(rJFH1 或 rJFH1/GND) 与由 Huh7 细胞提取的总 RNA 混合，使 RNA 量达到 10μg。随后，利用电穿孔法将混合的 RNA 导入到 Huh7 细胞中。将进行电穿孔处理的 Huh7 细胞接种入培养皿中。在温育 12、24、48 和 72 小时后，收集细胞，提取 RNA，并通过 RNA 印迹法分析。按照 Molecular Cloning, A laboratory Manual，第 2 版，J. Sambrook, E. F. Fritsch, T. Maniatis, Cold Spring Harbor Laboratory Press(1989) 进行 RNA 印迹分析。具体地说，对由温育后细胞提取的 RNA 进行变性琼脂糖凝胶电泳，并在电泳后将 RNA 转移至带正电荷的尼龙膜上。使由 pJFH1 制备的 32P 标记 DNA 或 RNA 探针与前述转移至膜上的 RNA 杂交。漂洗膜，并使膜对胶片曝光，以检测对 JFH-1 克隆的全长 HCV 基因组 RNA 特异性的 RNA 条带。

[0178] 如图 2 所示，当 rJFH1/GND 转染至细胞中时，在转染后 4 小时导入 RNA 的条带被证实为弱信号，但信号随着传代次数变得更弱，转染后 24 小时的条带信号几乎不可检测。相

比之下，当转染 rJFH1 时，导入 RNA 条带的信号强度首先变弱，和 rJFH1/GND 转染后 4-12 小时的情况一样，但在转染后 24 小时证实了清晰的 RNA 信号。证实的信号是 HCV 基因组 RNA 特异性的。也就是说，一般认为某些导入的全长 HCV 基因组 RNA 复制并生长。对于其中为 RNA 复制酶的 NS5B 活性基序突变的 rJFH1/GND，未观察到复制，这表明 NS5B 活性对全长 HCV 基因组 RNA 复制很重要。此外，对得自丙型肝炎病毒，例如 H77 毒株（非特许文献 7）、J6 毒株（非特许文献 8）和本发明发明人由慢性肝炎患者分离的 JCH1 毒株（非特许文献 6）的全长基因组 RNA（先前已全部被分离）进行相同的实验，但证实这些毒株的全长 HCV 基因组 RNA 未复制。

[0179] (D) 转染细胞培养物的培养基中 HCV 病毒颗粒的检测

[0180] 将如上所述的电穿孔处理的 Huh7 细胞接种在培养皿中，培养 12、24、48 和 72 小时，然后检测培养上清液中的 HCV 核心蛋白。按照 Ortho HCV 抗原 IRMA 检验（非特许文献 9）进行检测。如图 3 所示，在用 rJFH1 转染后 48 和 72 小时检测培养上清液中的核心蛋白。为检验此核心蛋白是否作为病毒颗粒被分泌，通过蔗糖密度梯度分级分离 rJFH1 转染后 72 小时的培养基。在离心管中，2ml 60％（重量／重量）蔗糖溶液（溶解在 50mM Tris pH 7.5/0.1M NaCl/1mM EDTA 中）、1ml 50％蔗糖溶液、1ml 40％蔗糖溶液、1ml 30％蔗糖溶液、1mlof 20％蔗糖溶液和 1ml 10％蔗糖溶液分层，4ml 样品培养上清液覆盖于其上。在 Beckman 转子 SW41 Ti 中以 400,000 RPM 于 4℃离心 16 小时。在离心后，由管底部收集各 0.5ml 的级分。测定各个级分中的密度、HCV 核心蛋白浓度和全长 HCV 基因组 RNA 的量。按照 Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-KoharaK, Kawaguchi R, Tanaka S, Kohara M,“Real-Time detection system forquantification of Hepatitis C virus genome”, Gastroenterology 116:636-642(1999)，通过检测全长 HCV 基因组 RNA 的 5′ 非翻译区的 RNA，用定量 RT-PCR 法进行全长 HCV 基因组 RNA 的检测。具体地说，使用合成引物 R6-130-S17:5′ -CGGGAGAGCCATAGTGG-3′ (SEQ IDNO:16)、R6-290-R19:5′ -AGTACCACAAGGCCTTTCG-3′ (SEQ ID NO:17) 和 TaqMan 探针:R6-148-S21FT, 5′ -CTGCGGAACCGGTGAGTACAC-3′ (SEQ ID NO:18) 以及 EZ rTthRNA PCR 试剂盒，PCR 扩增由细胞提取的 RNA 中包含的全长 HCV 基因组 RNA，然后通过 ABI Prism 7700 序列检测系统检测。

[0181] 如图 4 所示，在级分 11 中核心蛋白的峰与全长 HCV 基因组 RNA 的峰一致。该级分的密度约为 1.18mg/ml，其表明比重比迄今为止所报道的核心蛋白和核酸缀合物的比重低。此外，当在用 0.25％ NP40 处理培养上清液后进行相似的分级分离时，核心蛋白和全长 HCV 基因组 RNA 的峰迁移至约 1.28mg/ml 的比重。也就是说，一般认为 NP40 处理剥落了病毒颗粒的表面膜（其含脂质，于是具有较低比重），产生仅由核酸和核心蛋白组成的核心颗粒，因此比重增加。以上结果表明，通过将 rJFH1 转染至 Huh7 细胞中，全长 HCV 基因组 RNA 在细胞中复制，结果形成病毒颗粒，并分泌入培养上清液中。

[0182] 实施例 3

[0183] (E) 全长 HCV 复制子 RNA 复制型细胞的制备和细胞克隆的建立

[0184] 通过如实施例 2 所述，将实施例 1 中制备的 rFGREP-JFH1 和 rFGREP-JFH1/GND 转染入 Huh7 细胞，制备全长 HCV 复制子 RNA 复制型细胞，然后尝试建立全长 HCV 复制子 RNA 复制型细胞克隆。

[0185] 首先，在分别将 rFGREP-JFH1 和 rFGREP-JFH1/GND 转染入 Huh7 细胞中后，将细胞接种在培养皿中。在培养 16-24 小时后，加入不同浓度的 G418。继续培养，同时每周更换两次培养基。在培养 21 天后，用结晶紫染色存活细胞。计数染色的集落，计算获得的集落数 / 用于转染的 RNA 重量。对于某些培养皿又继续培养，以克隆存活细胞的集落。分别由克隆细胞中提取 RNA、基因组 DNA 和蛋白，然后检测全长 HCV 复制子 RNA，研究新霉素抗性基因在基因组 DNA 中的整合以及 HCV 蛋白的表达。这些结果在下文详细给出。

[0186] (F) 集落形成能力

[0187] 以上转染的结果表明，对于在 1.0mg/ml G418 浓度下用 rFGREP-JFH1 转染的 Huh7 细胞（图 5 的左部），集落形成能力 /1μg 用于转染的复制子 RNA 是 368 CFU（集落形成单位）/μg RNA。相反，对于用 rFGREP-JFH1/GND 转染的 Huh7 细胞（图 5 的右部），未观测到集落形成。这表明，用 rFGREP-JFH1 复制子 RNA 转染的 Huh7 细胞的集落形成能力依赖于由 rFGREP-JFH1 表达的 NS5B(RNA 聚合酶）的活性。也就是说，一般认为在集落形成细胞中，借助于由 rFGREP-JFH1 表达的 NS5B 的作用，rFGREP-JFH1 复制子 RNA 自主复制，引起新霉素抗性基因持续表达，因此保持了 G418 抗性，结果使细胞生长成为可能。

[0188] (G) 在已建立细胞克隆中的全长 HCV 复制子 RNA 的检测

[0189] 利用酸 - 苯酚提取法，由全长 HCV 复制子 RNA 复制型细胞克隆中提取总 RNA，该细胞按照以上章节 (E) 通过将 rFGREP-JFH1 转染入 Huh7 细胞中而建立。随后，利用 RNA 印迹法检测此总 RNA。在该方法中，使用 pFGREP-JFH1 特异性探针。以相似的方式由未转染的 Huh7 细胞提取的总 RNA（在图 6 中显示为“Huh7”），一个除由 Huh7 细胞提取的总 RNA 以外还含体外合成的 10^7 个拷贝的复制子 RNA 的样品（在图 6 中显示为“10^7”）和一个除由 Huh7 细胞提取的总 RNA 以外还含体外合成的 10^8 个拷贝的复制子 RNA 的样品（在图 6 中显示为“10^8”）用作对照。在图 6 中，1-4 表示细胞克隆号。

[0190] 结果，用 rFGREP-JFH1 特异性探针检测与 rFGREP-JFH1 具有相似大小的 RNA（图 6）。由该结果证实转染的 rFGREP-JFH1 复制子 RNA 在细胞克隆中复制和生长。还表明细胞克隆中的复制子 RNA 的量有差异。如图 6 所示，例如，克隆 2 中的复制子 RNA 的量少于其它克隆。

[0191] (H) 验证新霉素抗性基因有无整合入基因组 DNA 中

[0192] 对于按照 (E) 所获得的细胞克隆 1-8（如图 7 中的 FGR-JFH1/2-1 至 FGR-JFH1/2-8 所示），使用新霉素抗性基因特异性引物（有义引物 NEO-S3：5′-AACAAGATGGATTGCACGCA-3′ (SEQ ID NO：19)，反义引物 NEO-R：5′-CGTCAAGAAGGCGATAGAAG-3′ (SEQ ID NO：20)），并使用由每个细胞克隆提取的宿主基因组 DNA 作为模板，进行 PCR 扩增，以便证实各个细胞克隆抗 G418 的抗性并非是由于新霉素抗性基因整合入宿主细胞基因组中。结果，如图 7 所示，未观测到显示扩增新霉素抗性基因的阳性克隆。

[0193] (H) 的结果证实，全长 HCV 复制子 RNA 在通过本发明的全长 HCV 复制子 RNA 转染建立的细胞克隆中复制。

[0194] (I)HCV 蛋白的检测

[0195] 通过标准方法由通过 rFGREP-JFH1 转染建立的细胞克隆提取蛋白，然后通过 SDS-PAGE 和蛋白质印迹法分析。在此情况下检测的细胞克隆与以上章节 (G) 中使用的克

隆相同。通过将制备的全长 HCV 基因组 RNA 瞬时转染入 Huh7 细胞中获得细胞提取物，将其用作阳性对照（在图 8、9 和 10 中显示为 JFH-1）。通过转染 HCV 亚基因组 RNA 复制子（SGR-JFH1）获得的克隆的细胞提取物用作核心蛋白的阴性对照，并用作 NS3 和 NS5a 蛋白的阳性对照（在图 8、9 和 10 中显示为 SGR-JFH1）。未转染的 Huh7 细胞的细胞提取物用作所有蛋白的阴性对照（在图 8、9 和 10 中显示为 Huh7）。将由各个细胞克隆提取的蛋白样品印迹在 PVDF 膜（Immobilon-P，Millipore）上，然后使用抗核心蛋白特异性抗体和抗 NS3 特异性抗体（由 Dr. Moradpour 惠赠；Wolk B 等，J. Virology，2000，74：2293-2304）检测由其中的全长 HCV 复制子 RNA 编码的核心蛋白和 NS3 蛋白。如图 8 和 9 所示，对于通过 rFGREP-JFH1 转染建立的细胞克隆 1-4，检测各个蛋白中与阳性对照大小相同的蛋白。因为在未转染 Huh7 细胞中既没有检测到核心蛋白，也没有检测到 NS3 蛋白，所以证实在细胞克隆 1-4 中已转染的全长 HCV 复制子 RNA 自主复制，表达核心蛋白和 NS3 蛋白。

[0196] 此外，对于每个细胞克隆，已如上所述检验 NS3 蛋白的表达，还使用丙型肝炎患者的血清作为抗体检验由全长 HCV 复制子 RNA 表达的 NS5A 蛋白（图 10）。

[0197] 由上述 (H) 和 (I) 的结果证实，在已通过全长 HCV 复制子 RNA 转染建立的细胞克隆中，全长 HCV 复制子 RNA 复制，病毒蛋白也表达。

[0198] (J) 全长 HCV 复制子 RNA 复制型细胞中的病毒颗粒生产

[0199] 按照以上章节 (E) 将 rFGREP-JFH1 转染入 Huh7 细胞中，建立全长 HCV 复制子 RNA 复制型细胞克隆 2 和 3(FGR-JFH1/2-3)，然后回收其培养上清液。按照与上述 (D) 相似的方法检测培养上清液中的 HCV 病毒颗粒。结果示于图 11。在图 11 中，阴影圆代表各个级分的比重 (g/ml)。实心圆代表核心蛋白的量 (fmol/L)。空心圆代表全长 HCV 复制子 RNA 的滴度（×0.1 拷贝 /mL）。

[0200] 如图 11 所示，核心蛋白峰与比重约 1.18-1.20mg/ml 的级分中的全长 HCV 复制子 RNA 的峰一致。还在较轻级分中发现小峰。以上结果表明，全长 HCV 复制子 RNA 在用 rFGREP-JFH11 转染的 Huh7 细胞中复制，形成病毒颗粒并分泌入其培养上清液中。

[0201] 实施例 4

[0202] (K) 采用培养上清液中的病毒颗粒的感染实验

[0203] 通过将在 (H) 中使用的细胞克隆 1-8（即 FGR-JFH1/2-1、FGR-JFH1/2-2、FGR-JFH1/2-3、FGR-JFH1/2-4、FGR-JFH1/2-5、FGR-JFH1/2-6、FGR-JFH1/2-7、FGR-JFH1/2-8）的各个培养上清液加入 Huh7 细胞中，用在培养上清液中的病毒颗粒感染 Huh7 细胞。在第 2 天，以 0.3mg/ml 向感染的 Huh7 细胞的培养基中加入 G418，再培养 Huh7 细胞 21 天。在结束培养后，固定细胞，并用结晶紫染色。对于分别用 FGR-JFH1/2-3、FGR-JFH1/2-5 和 FGR-JFH1/2-6 的培养上清液感染的细胞，观测到集落形成。另一方面，对于用作对照的亚基因组复制子细胞 SGR-JFH1/4-1（描述于非特许文献 6）的培养上清液感染的细胞，未观测到集落形成。图 12 显示了与加入的 4ml 或 8mlFGR-JFH1/2-3 或 SGR-JFH1/4-1 培养上清液培养 21 天后染色培养皿的照片。在其中接种与 4ml 或 8ml FGR-JFH1/2-3 培养上清液混合的细胞的培养皿中，分别发现 3 个和 9 个集落。但是，在其中接种与 SGR-JFH1/4-1 的培养上清液混合的细胞的培养皿中，未观测到集落。

[0204] 随后，克隆分别使用 FGR-JFH1/2-3 和 FGR-JFH1/2-5 的培养上清液以丙型肝炎病毒感染形成的集落。由用 FGR-JFH1/2-3 的培养上清液感染的培养皿建立 3 个克隆

FGR-JFH1/C2-3-11、FGR-JFH1/C2-3-12 和 FGR-JFH1/C2-3-13，并由用 FGR-JFH1/C2-5 的培养上清液感染的培养皿建立 2 个克隆 FGR-JFH1/C2-5-11 和 FGR-JFH1/C2-5-12。

[0205] 当 用 FGR-JFH1/C2-3-11、FGR-JFH1/C2-3-12、FGR-JFH1/C2-3-13、FGR-JFH1/C2-5-11 和 FGR-JFH1/C2-5-12 各个细胞克隆的培养上清液感染 Huh7 细胞时，在分别用 FGR-JFH1/C2-3-12 和 FGR-JFH1/C2-5-12 的培养上清液感染的培养皿中观测到集落形成。由用 FGR-JFH1/C2-3-12 的培养上清液感染的细胞建立另两个克隆 FGR-JFH1/C2-3-12-1 和 FGR-JFH1/C2-3-12-2。由用 FGR-JFH1/C2-5-12 的培养上清液感染的细胞建立另两个克隆 FGR-JFH1/C2-5-12-1 和 FGR-JFH1/C2-5-12-2。

[0206] 已用全长 HCV 复制子 RNA 复制型细胞的培养上清液感染的细胞建立细胞，由这些细胞克隆提取 RNA、蛋白和基因组 DNA。通过使用基因组 DNA 作为模板的 PCR，检验新霉素抗性基因在这些细胞克隆的基因组 DNA 中的整合，结果全为阴性。而且，通过使用 RNA 作为模板的定量 PCR 可检测细胞中复制的全长 HCV 复制子 RNA。再进一步，可检测培养上清液中的核心蛋白。这些结果表明，由本发明的全长 HCV 复制子 RNA 复制型细胞生产的含全长 HCV 复制子 RNA 的病毒颗粒可感染另一细胞。

[0207] 工业实用性

[0208] 按照本发明的方法，可在细胞培养系统中制备 HCV 病毒颗粒。使用本发明的复制子 RNA，可在细胞培养系统中有效生产含全长 HCV 基因组 RNA 的 RNA。而且，通过使用其中导入本发明的全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 的细胞，全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 可在细胞培养系统中复制，本发明的 HCV 病毒颗粒可在细胞培养系统中连续生产。其中导入本发明的全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 的细胞还可用作测试系统，用于筛选影响 HCV 复制过程、病毒颗粒形成过程和病毒颗粒胞外释放过程的各种物质。本发明的全长 HCV 复制子 RNA 和全长 HCV 基因组 RNA 以及病毒颗粒还用作外源基因的病毒载体。本发明的病毒颗粒或其部分可包含在疫苗中，作为抗丙型肝炎病毒的疫苗抗原。此外，其中本发明的病毒颗粒和其它细胞一起培养的系统可用作测试系统，用于筛选对病毒颗粒感染细胞有影响的各种物质。本发明的全长 HCV 复制子 RNA 或全长 HCV 基因组 RNA 用作模板，该模板能使 HCV 全长基因组序列简单再生。

[0209] 本文提及的所有出版物、特许和特许申请都全文在此引入作为参考。

[0210] 无内容序列表

[0211] SEQ ID NO :1 代表来自 JFH-1 克隆的 HCV 基因组 RNA 的 5′ 非翻译区序列。

[0212] SEQ ID NO :2 代表来自 JFH-1 克隆的 HCV 基因组 RNA 的核心蛋白编码序列。

[0213] SEQ ID NO :3 代表来自 JFH-1 克隆的 HCV 基因组 RNA 的 E1 蛋白编码序列。

[0214] SEQ ID NO :4 代表来自 JFH-1 克隆的 HCV 基因组 RNA 的 E2 蛋白编码序列。

[0215] SEQ ID NO :5 代表来自 JFH-1 克隆的 HCV 基因组 RNA 的 NS2 蛋白编码序列。

[0216] SEQ ID NO :6 代表来自 JFH-1 克隆的 HCV 基因组 RNA 的 NS3 蛋白编码序列。

[0217] SEQ ID NO :7 代表来自 JFH-1 克隆的 HCV 基因组 RNA 的 NS4A 蛋白编码序列。

[0218] SEQ ID NO :8 代表来自 JFH-1 克隆的 HCV 基因组 RNA 的 NS4B 蛋白编码序列。

[0219] SEQ ID NO :9 代表来自 JFH-1 克隆的 HCV 基因组 RNA 的 NS5A 蛋白编码序列。

[0220] SEQ ID NO :10 代表来自 JFH-1 克隆的 HCV 基因组 RNA 的 NS5B 蛋白编码序列。

[0221] SEQ ID NO :11 代表来自 JFH-1 克隆的 HCV 基因组 RNA 的 3′ 非翻译区序列。

[0222] SEQ ID NO:12 代表来自 JFH-1 克隆的全长 HCV 基因组 RNA 序列。

[0223] SEQ ID NO:13 代表包含来自 JFH-1 克隆的全长 HCV 基因组 RNA 的复制子 RNA 序列。

[0224] SEQ ID NO:14 代表来自 JFH-1 克隆的全长 HCV 基因组 RNA 序列,其中氨基酸基序 GDD 已突变为 GND。

[0225] SEQ ID NO:15 代表包含来自 JFH-1 克隆的全长 HCV 基因组 RNA 的复制子 RNA 序列,其中氨基酸基序 GDD 已突变为 GND。

[0226] SEQ ID NO:16-20 代表引物序列。

[0227] SEQ ID NO:21 代表来源于表达载体 pFGREP-JFH1/Luc 的复制子 RNA 的序列。

[0228] SEQ ID NO:22 代表来源于表达载体 pFGREP-JFH1/Luc/GND 的复制子 RNA 的序列。

[0229] SEQ ID NO:23 代表来源于表达载体 pFGREP-JFH1/EGFP 的复制子 RNA 的序列。

[0230] SEQ ID NO:24 代表来源于表达载体 pFGREP-JFH1/EGFP/GND 的复制子 RNA 的序列。

[0231] SEQ ID NO:25 代表来源于表达载体 pFGREP-JFH1/SEAP 的复制子 RNA 的序列。

[0232] SEQ ID NO:26 代表来源于表达载体 pFGREP-JFH1/SEAP/GND 的复制子 RNA 的序列。

[0001]

序 列 表

<110> 东京都医学研究机构
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<120> 含人丙型肝炎病毒全长基因组的核酸构建物、核酸构建物转入其中的重组全长病毒基因组复制型细胞和生产人丙型肝炎病毒颗粒的方法

<130> PH-2372-PCT

<140> PCT/JP2005/003232
<141> 2005-02-21

<150> JP 2004-045489
<151> 2004-02-20

<160> 26

<170> PatentIn Ver. 2.1

<210> 1
<211> 340
<212> RNA
<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的丙型肝炎病毒基因组 RNA 的 5′非翻译区

<220>
<223> 发明人：Wakita, Takaji
发明人：Kato, Takanobu
发明人：Date, Tomoko
发明人：Miyamoto, Michiko
发明人：Tanabe, Junichi
发明人：Sone, Saburo

<400> 1
accugccccu aauaggggcg acacuccgcc augaaucacu ccccugugag gaacuacugu 60
cuucacgcag aaagcgccua gccauggcgu uaguaugagu gucguacagc cuccaggccc 120
cccccucccg ggagagccau aguggucugc ggaaccggug aguacaccgg aauugccggg 180
aagacugggu ccuuucuugg auaaacccac ucuaugcccg gccauuuggg cgugcccccg 240
caagacugcu agccgaguag cguuggguug cgaaaggccu ugugguacug ccugauaggg 300
cgcuugcgag ugccccggga ggucucguag accgugcacc 340

<210> 2
<211> 573

[0002]

<212> RNA
<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的丙型肝炎病毒基因组 RNA 的核心蛋白编码序列

<400> 2
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gacguuaagu ucccgggcgg cggccagauc guuggcggag uauacuuguu gccgcgcagg 120
ggccccaggu ugggugugcg cacgacaagg aaaacuucgg agcgguccca gccacguggg 180
agacgccagc ccaucccccaa agaucggcgc uccacuggca aggccugggg aaaaccaggu 240
cgccccuggc cccuauauugg gaaugaggga cucggcuggg caggauggcu ccugucccccc 300
cgaggcucuc gccccuccug gggccccacu gacccccggc auaggucgcg caacgugggu 360
aaagucaucg acacccuaac guguggcuuu gccgaccuca ugggguacau ccccgucgua 420
ggcgccccgc uuaguggcgc cgccagagcu gucgcgcacg gcgugagagu ccuggaggac 480
gggguuaauu augcaacagg gaaccuaccc gguuuccccu uuucuaucuu cuugcuggcc 540
cuguuguccu gcaucaccgu uccggucucu gcu 573

<210> 3
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<212> RNA
<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的丙型肝炎病毒基因组 RNA 的 E1 蛋白编码序列

<400> 3
gcccagguga agaauaccag uagcagcuac auggugacca augacugcuc caaugacagc 60
aucacuuggc agcucgaggc ugcgguucuc cacgucccccg ggugcgucccc gugcgagaga 120
gugggggaaua cgucacggug uugggugcca gucucgccaa acauggcugu gcggcagccc 180
ggugcccuca cgcagggucu gcggacgcac aucgauaugg uugugauguc cgccaccuuc 240
ugcucugcuc ucuacguggg ggaccucugu ggcgggguga ugcucgcggc ccagguguuc 300
aucgucucgc cgcaguacca cugguuugug caagaaugca auugcuccau cuacccuggc 360
accaucacug gacaccgcau ggcaugggac augaugauga acuggucgcc cacggcccacc 420
augauccugg cguacgugau gcgcgucccc gaggucauca uagacaucgu uagcggggcu 480
cacuggggcg ucauguucgg cuuggccuac uucucuaugc agggagcgug ggcgaagguc 540
auugucaucc uucugcuggc cgcuggggug gacgcg 576

<210> 4
<211> 1290
<212> RNA
<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的丙型肝炎病毒基因组 RNA 的 E2 蛋白编码序列

<400> 4

[0003]

ggcaccacca ccguuggagg cgcuguugca cguuccacca acgugauugc cggcguguuc 60
agccauggcc cucagcagaa cauucagcuc auuaacacca acggcaguug gcacaucaac 120
cguacugccu ugaauugcaa ugacuccuug aacaccggcu uucucgcggc cuuguucuac 180
accaaccgcu uuaacucguc agggugucca gggcgccugu ccgccugccg caacaucgag 240
gcuuuccgga uagggugggg cacccuacag uacgaggaua augucaccaa uccagaggau 300
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acccgaccgc cgcagggcuc augguucggc ugcacgugga ugaacuccac ugguuucacc 540
aagacuugug gcgcgccacc uugccgcacc agagcugacu ucaacgccag cacggacuug 600
uugugcccua cggauuguuu uaggaagcau ccugaugcca cuuauauuaa gugugguucu 660
gggcccuggc ucacaccaaa gugccugguc cacuacccuu acagacucug gcauuacccc 720
ugcacaguca auuuuaccau cuucaagaua agaauguaug uagggggggu ugagcacagg 780
cucacggccg caugcaacuu cacucguggg gaucgcugcg acuuggagga cagggacagg 840
agucagcugu cuccucuguu gcacucuacc acggaauggg ccauccugcc cugcaccuac 900
ucagacuuac ccgcuuuguc aacuggucuu cuccaccuuc accagaacau cguggacgua 960
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cucauggcac ugccccggca ggcuuaugcc 1290

<210> 5
<211> 651
<212> RNA
<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的丙型肝炎病毒基因组 RNA 的 NS2 蛋白编码序列

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acacucaccc cgggguauaa gacccuccuc ggccaguguc uguggugguu gugcuaucuc 120
cugacccugg gggaagccau gauucaggag uggguaccac ccaugcaggu gcgcggcggc 180
cgcgauggca ucgcgugggc cgucacuaua uucugccccgg gugugguguu ugacauuacc 240
aaauggcuuu uggcguugcu ugggccugcu uaccucuuaa gggccgcuuu gacacaugug 300
ccguacuucg ucagagcuca cgcucugaua aggguaugcg cuuuggugaa gcagcucgcg 360
gggguaggu auguucaggu ggcgcuauug gcccuuggca gguggacugg caccuacauc 420
uaugaccacc ucacaccuau gucggacugg gccgcuagcg gccugcgcga cuuagcgguc 480
gccguggaac ccaucaucuu caguccgaug gagaagaagg ucaucgucug gggagcggag 540
acggcugcau gugggacau ucuacaugga cuucccgugu ccgcccgacu cggccaggag 600
auccucccucg gcccagcuga uggcuacacc uccaaggggu ggaagcuccu u 651

<210> 6
<211> 1893
<212> RNA

[0004]

<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的丙型肝炎病毒基因组 RNA 的 NS3 蛋白编码序列

<400> 6

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augacggggc gugacaggac agaacaggcc ggggaagucc aaauccuguc cacagucucu 120
caguccuucc ucggaacaac caucucgggg guuuugugga cuguuuacca cggagcuggc 180
aacaagacuc uagccggcuu acgggguccg gucacgcaga uguacucgag ugcugagggg 240
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gccgucgacc uauaucuggu cacgcggaac gcugauguca ucccggcucg gagacgcggg 360
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ucucccacuu ucagugacaa cagcacgcca ccggcugugc cccagaccua ucaggucggg 600
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auguggaagu gccuggcccg acucaagccu acgcuugcgg gccccacacc ucuccuguac 1800
cguuugggcc cuauuaccaa ugaggucacc cucacacacc cugggacgaa guacaucgcc 1860
acaugcaugc aagcugaccu ugaggucaug acc 1893

<210> 7
<211> 162
<212> RNA
<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的丙型肝炎病毒基因组 RNA 的 NS4A 蛋白编码序列

<400> 7

[0005]

agcacguggg uccuagcugg aggaguccug gcagccgucg ccgcauauug ccuggcgacu 60
ggaugcguuu ccaucaucgg ccgcuugcac gucaaccagc gagucgucgu ugcgccggau 120
aaggaggucc uguaugaggc uuuugaugag auggaggaau gc 162

<210> 8
<211> 783
<212> RNA
<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的丙型肝炎病毒基因组 RNA 的 NS4B 蛋白编码序列

<400> 8
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auccaaggcu ugcugcagca ggccucuaag caggcccagg acauacaacc cgcuaugcag 120
gcuucauggc ccaaagugga acaauuuugg gccagacaca uguggaacuu cauuagcggc 180
auccaauacc ucgcaggauu gucaacacug ccagggaacc ccgcgguggc uuccaugaug 240
gcauucagug ccgcccucac caguccguug ucgaccagua ccaccauccu ucucaacauc 300
augggaggcu gguuagcguc ccagaucgca ccacccgcgg gggccaccgg cuuugucguc 360
aguggccugg ugggggcugc cgugggcagc auaggccugg guaaggugcu gguggacauc 420
cuggcaggau auggugcggg cauuucgggg gcccucgucg cauucaagau caugucuggc 480
gagaagcccu cuauggaaga ugucaucaau cuacugccug ggauccuguc uccgggagcc 540
cugguggugg gggucaucug cgcggccauu cugcgccgcc acgugggacc gggggagggc 600
gcgguccaau ggaugaacag gcuuauugcc uuugcuucca gaggaaacca cgucgccccu 660
acucacuacg ugacggaguc ggaugcgucg cagcguguga cccaacuacu uggcucucuu 720
acuauaacca gccuacucag aagacuccac aauuggauaa cugaggacug ccccauccca 780
ugc 783

<210> 9
<211> 1398
<212> RNA
<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的丙型肝炎病毒基因组 RNA 的 NS5A 蛋白编码序列

<400> 9
uccggauccu ggcuccgcga cgugugggac ugggguuugca ccaucuugac agacuucaaa 60
aauuggcuga ccucuaaauu guucccccaag cugcccggcc ucccccuucau cucuugucaa 120
aagggguaca agggugugug ggccggcacu ggcaucauga ccacgcgcug cccuugcggc 180
gccaacaucu cuggcaaugu ccgccugggc ucuaugagga ucacagggcc uaaaaccugc 240
augaacaccu ggcaggggac cuuuccuauc aauugcuaca cggagggcca gugcgcgccg 300
aaacccccca cgaacuacaa gaccgccauc uggagggugg cggccucgga guacgcggag 360
gugacgcagc augggucgua cuccuaugua acaggacuga ccacugacaa ucugaaaauu 420
ccuugccaac uaccuucucc agaguuuuuc uccugggugg acggugugca gauccauagg 480
uuugcaccca caccaaagcc guuuuuccgg gaugaggucu cguucugcgu ugggcuuaau 540
uccuaugcug ucggguccca gcuucccugu gaaccugagc ccgacgcaga cguauugagg 600

[0006]

uccaugcuaa cagauccgcc ccacaucacg gcggagacug cggcgcggcg cuuggcacgg 660
ggaucaccuc caucugaggc gagcuccuca gugagccagc uaucagcacc gucgcugcgg 720
gccaccugca ccacccacag caacaccuau gacguggaca uggucgaugc caaccugcuc 780
auggagggcg guguggcuca gacagagccu gaguccaggg ugcccguucu ggacuuucuc 840
gagccaaugg ccgaggaaga gagcgaccuu gagcccucaa uaccaucgga gugcaugcuc 900
cccaggagcg gguuuccacg ggccuuaccg gcuugggcac ggccugacua caacccgccg 960
cucguggaau cguggaggag gccagauuac caaccgccca ccguugcugg uugugcucuc 1020
ccccccccca agaaggcccc gacgccuccc ccaaggagac gccggacagu gggucugagc 1080
gagagcacca uaucagaagc ccuccagcaa cuggccauca agaccuuugg ccagcccccc 1140
ucgagcggug augcaggcuc guccacgggg gcgggcgccg ccgaauccgg cgguccgacg 1200
uccccuggug agccggcccc cucagagaca gguuccgccu ccucuaugcc ccccccucgag 1260
ggggagccug gagauccgga ccuggagucu gaucagguag agcuucaacc ucccccccag 1320
gggggggggg uagcucccgg uucgggcucg gggucuuggu cuacuugcuc cgaggaggac 1380
gauaccaccg ugugcugc 1398

<210> 10
<211> 1773
<212> RNA
<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的丙型肝炎病毒基因组 RNA 的 NS5B 蛋白编码序列

<400> 10
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uugccaauca acccuuugag uaacucgcug uugcgauacc auaacaaggu guacuguaca 120
acaucaaaga gcgccucaca gagggcuaaa aagguaacuu uugacaggac gcaagugcuc 180
gacgcccauu augacucagu cuuaaaggac aucaagcuag cggcuuccaa ggucagcgca 240
aggcuccuca ccuuggagga ggcgugccag uugacuccac cccauucugc aagauccaag 300
uauggauucg gggccaagga gguccgcagc uuguccggga gggccguuaa ccacaucaag 360
uccgugugga aggaccuccu ggaagaccca caaacaccaa uucccacaac caucauggcc 420
aaaaaugagg uguucugcgu ggaccccgcc aaggggggua agaaaccagc ucgccucauc 480
guuuacccug accucggcgu ccgggucugc gagaaaaugg cccucuauga cauuacacaa 540
aagcuuccuc aggcgguaau gggagcuucc uauggcuucc aguacucccc ugcccaacgg 600
guggaguauc ucuugaaagc augggcggaa aagaaggacc ccauggguuu uucguaugau 660
acccgaugcu ucgacucaac cgucacugag agagacauca ggaccgagga guccauauac 720
caggccugcu cccugcccga ggaggcccgc acugccauac acucgcugac ugagagacuu 780
uacguaggag ggcccauguu caacagcaag ggucaaaccu gcgguuacag acguugccgc 840
gccagcgggg ugcuaaccac uagcaugggu aacaccauca caugcuaugu gaaagcccua 900
gcggccugca aggcugcggg gauaguugcg cccacaaugc ugguaugcgg cgaugaccua 960
guagucaucu cagaaagcca ggggacugag gaggacgagc ggaaccugag agccuucacg 1020
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[0007]

gaacugacgc ggguggcuuc agcccucaga aaacuugggg cgccaccccu cagggugugg 1500
aagagucggg cucgcgcagu cagggcgucc cucaucuccc guggagggaa agcggccguu 1560
ugcggccgau aucucuucaa uugggcggug aagaccaagc ucaaacucac uccauugccg 1620
gaggcgcgcc uacuggacuu auccaguugg uucaccgucg gcgccggcgg gggcgacauu 1680
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<210> 11
<211> 239
<212> RNA
<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的丙型肝炎病毒基因组 RNA 的 3′非翻译区

<400> 11
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ucuuauucua cuuucuuucu ugguggcucc aucuuagccc uagucacggc uagcugugaa 180
aggucccguga gccgcaugac ugcagagagu gccguaacug gucucucugc agaucaugu 239

<210> 12
<211> 9707
<212> RNA
<213> 丙型肝炎病毒

<220>
<223> 来自 JFH-1 克隆的全长丙型肝炎病毒基因组 RNA

<400> 12
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cacuccccug ugaggaacua cugucuucac gcagaaagcg ccuagccaug gcguuaguau 120
gagugucgua cagccuccag gccccccccu cccgggagag ccauaguggu cugcggaacc 180
ggugaguaca ccggaauugc cgggaagacu ggguccuuuc uuggauaaac ccacucuaug 240
cccggccauu ugggcgugcc cccgcaagac ugcuagccga guagcguugg guugcgaaag 300
gccuuguggu acugccugau agggcgcuug cgagugcccc gggaggucuc guagaccgug 360
caccaugagc acaaauccua aaccucaaag aaaaaccaaa agaaacacca accgucgccc 420
agaagacguu aaguucccgg gcggcggcca gaucguuggc ggaguauacu uguugccgcg 480
caggggcccc agguugggug ugcgcacgac aaggaaaacu ucggagcggu cccagccacg 540
ugggagacgc cagcccaucc ccaaagaucg gcgcuccacu ggcaaggccu ggggaaaacc 600
aggucgcccc uggccccuau augggaauga gggacucggc ugggcaggau ggcuccuguc 660
ccccccgaggc ucucgccccu ccuggggccc cacugacccc cggcauaggu cgcgcaacgu 720
ggguaaaguc aucgacaccc uaacgugugg cuuugccgac cucauggggu acaucccccgu 780
cguaggcgcc ccgcuuagug gcgccgccag agcugucgcg cacggcguga gaguccugga 840
ggacgggguu aauuaugcaa cagggaaccu acccgguuuc cccuuuucua ucuucuugcu 900
ggcccuguug uccugcauca ccguuccggu cucugcugcc caggugaaga auaccaguag 960
cagcuacaug gugaccaaug acugcuccaa ugacagcauc acuuggcagc ucgaggcugc 1020

[0008]

gguucuccac gucccccgggu gcgucccgug cgagagagug gggaauacgu cacgguguug 1080
ggugccaguc ucgccaaaca uggcugugcg gcagcccggu gcccucacgc agggucugcg 1140
gacgcacauc gauaugguug ugauguccgc caccuucugc ucugcucucu acgugggggа 1200
ccucuguggc ggggugaugc ucgcggccca gguguucauc gucucgccgc aguaccacug 1260
guuugugcaa gaaugcaauu gcuccaucua cccuggcacc aucacuggac accgcauggc 1320
augggacaug augaugaacu ggucgcccac ggccaccaug auccuggcgu acgugaugcg 1380
cgucccccgag gucaucauag acaucguuag cggggcucac uggggcguca uguucggcuu 1440
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ugggguggac gcgggcacca ccaccguugg aggcgcuguu gcacguucca ccaacgugau 1560
ugccggcgug uucagccaug gcccucagca gaacauucag cucauuaaca ccaacggcag 1620
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ggccuuguuc uacaccaacc gcuuuaacuc gucagggugu ccagggcgcc uguccgccug 1740
ccgcaacauc gaggcuuucc ggauagggug gggcacccua caguacgagg auaaugucac 1800
caauccagag gauaugaggc cguacugcug gcacuacccc ccaaagccgu guggcguagu 1860
ccccgcgagg ucugugugug gcccagugua cuguuucacc cccagcccgg uaguaguggg 1920
cacgaccgac agacguggag ugcccaccua cacaugggga gagaaugaga cagaugucuu 1980
ccuacugaac agcacccgac cgccgcaggg cucaugguuc ggcugcacgu ggaugaacuc 2040
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[0015]

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[0019]

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[0020]

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[0022]

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<223> 人工序列的描述：引物

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<210> 17
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[0023]

<400> 17
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[0024]

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uuuuuuuuuu uuuuuuuuuu uucuuuuuuu uuuuuuuccc ucuuucuucc cuucucaucu 11760
uauucuacuu ucuuucuugg uggcuccauc uuagcccuag ucacggcuag cugugaaagg 11820
uccgugagcc gcaugacugc agagagugcc guaacugguc ucucugcaga ucaugu 11876

图 1

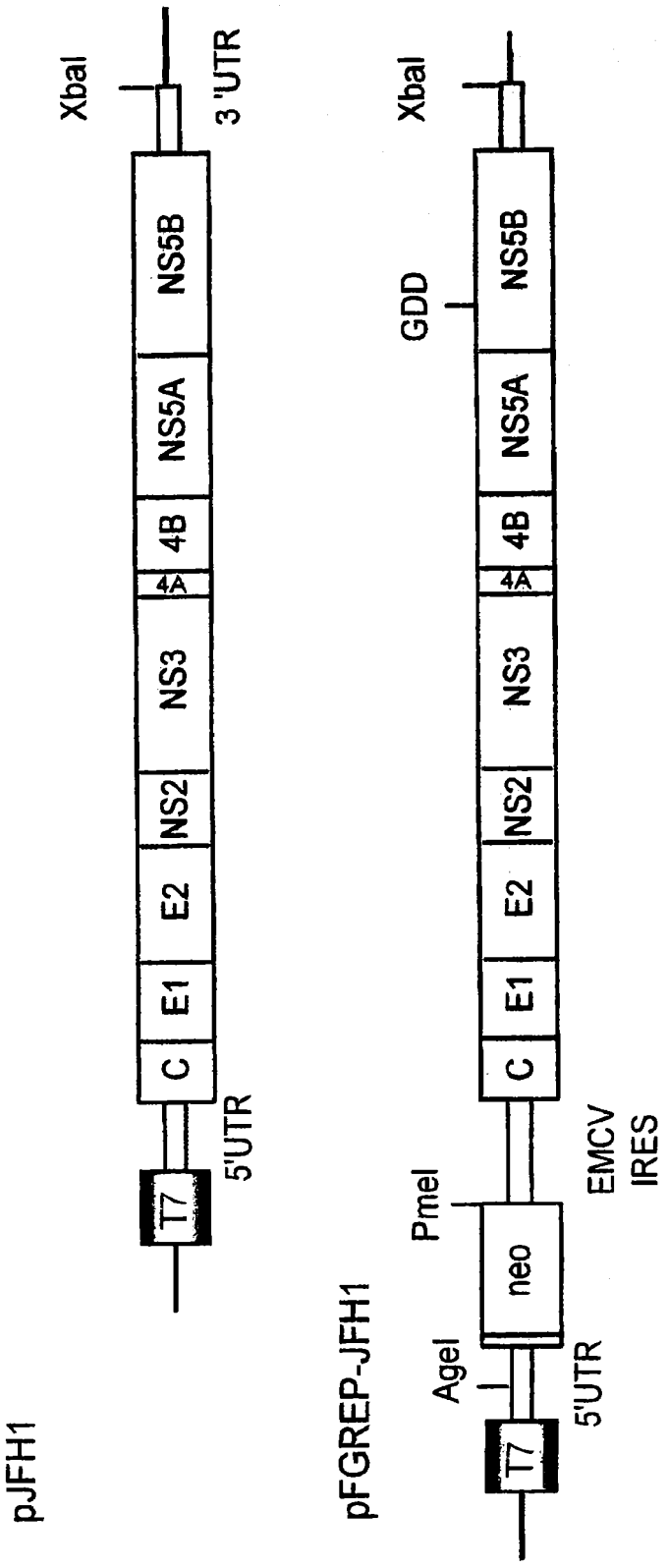

pJFH1
T7
5'UTR
C
E1
E2
NS2
NS3
4A
4B
NS5A
NS5B
3'UTR
XbaI
pFGREP-JFH1
T7
5'UTR
AgeI
neo
PmeI
EMCV
IRES
C
E1
E2
NS2
NS3
4A
4B
NS5A
NS5B
GDD
XbaI

图 2

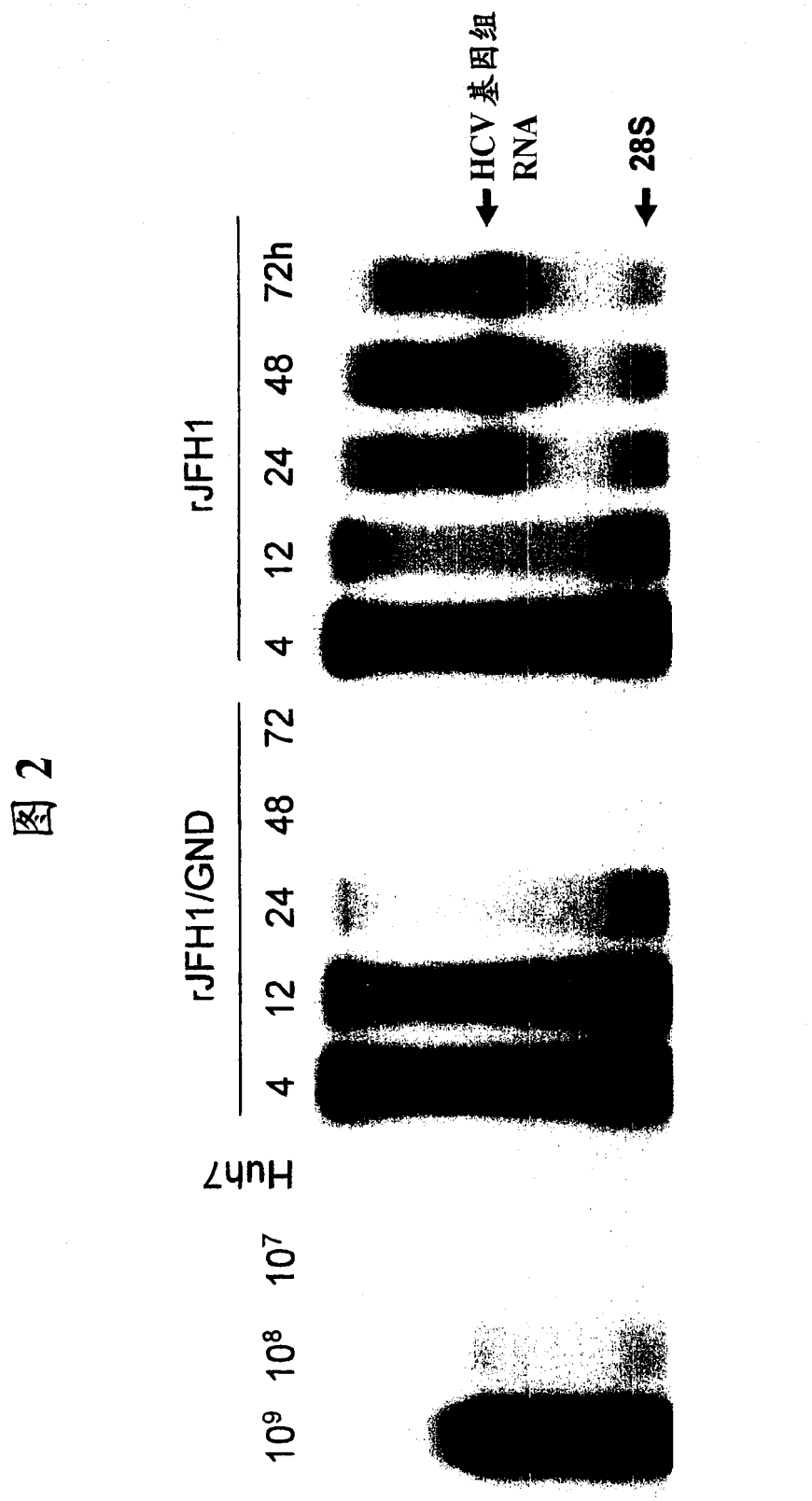

HCV 基因组 RNA
28S
rJFH1
rJFH1/GND
4
12
24
48
72h
72
Huh7
10^9
10^8
10^7

图 3

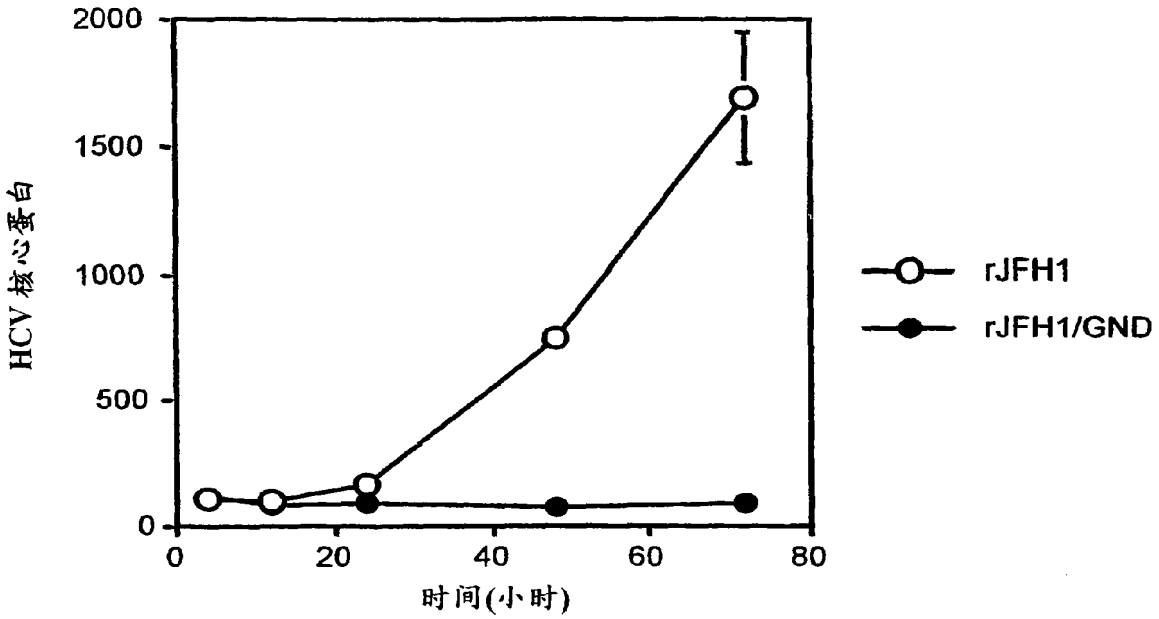


图 4

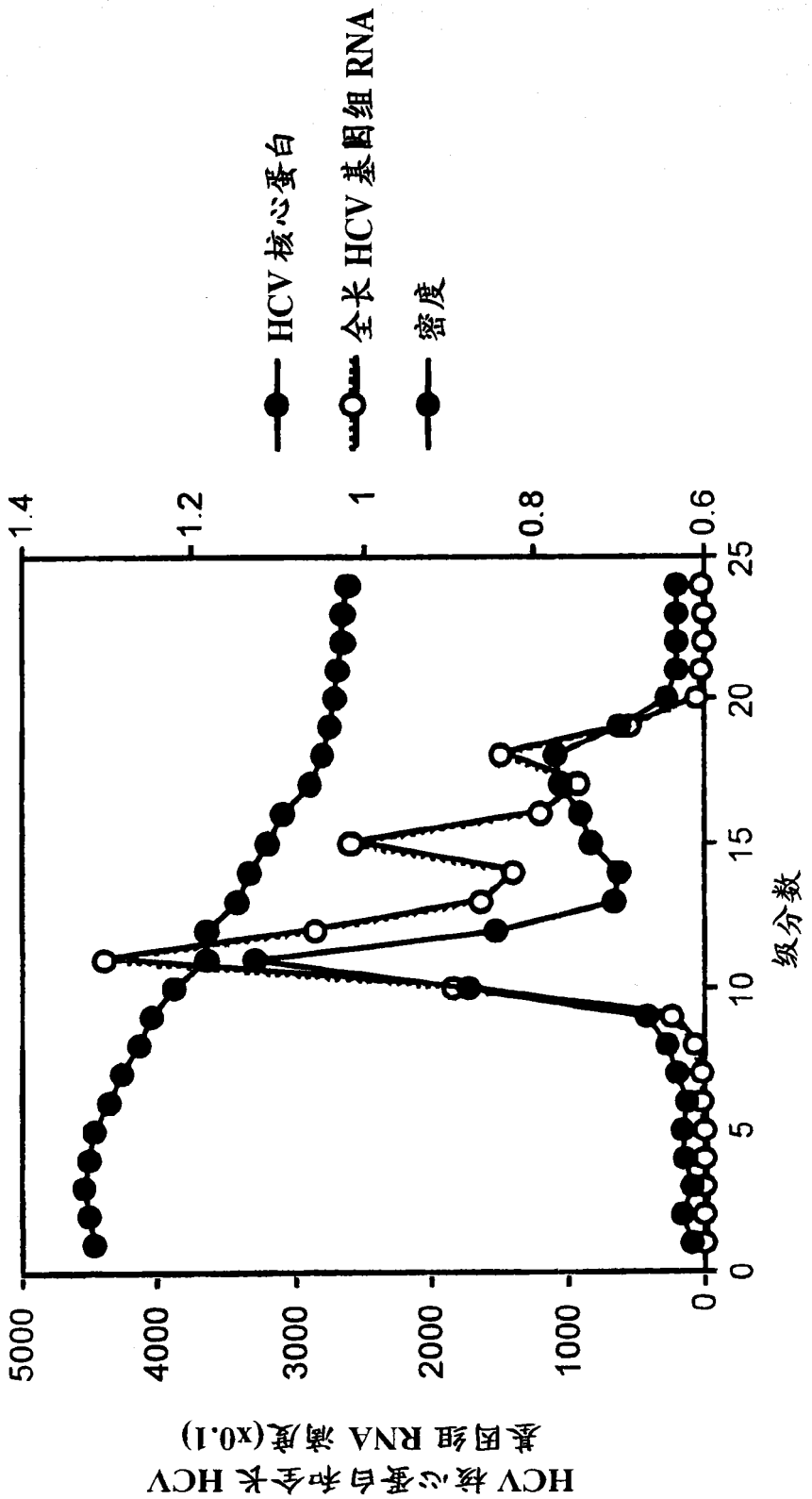

HCV 核心蛋白
全长 HCV 基因组 RNA
密度
HCV 核心蛋白和全长 HCV
基因组 RNA 滴度(x0.1)
级分数
5000
4000
3000
2000
1000
0
1.4
1.2
1
0.8
0.6
0
5
10
15
20
25

图 5

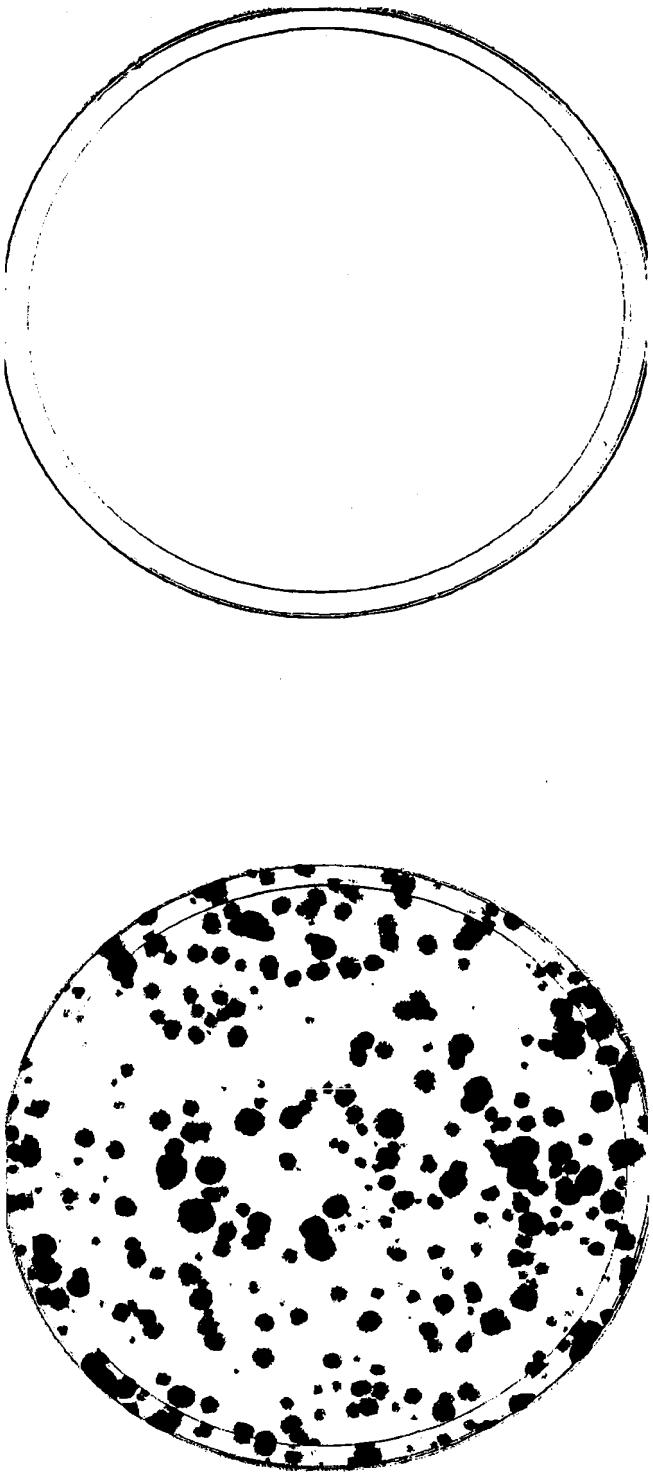


图 6

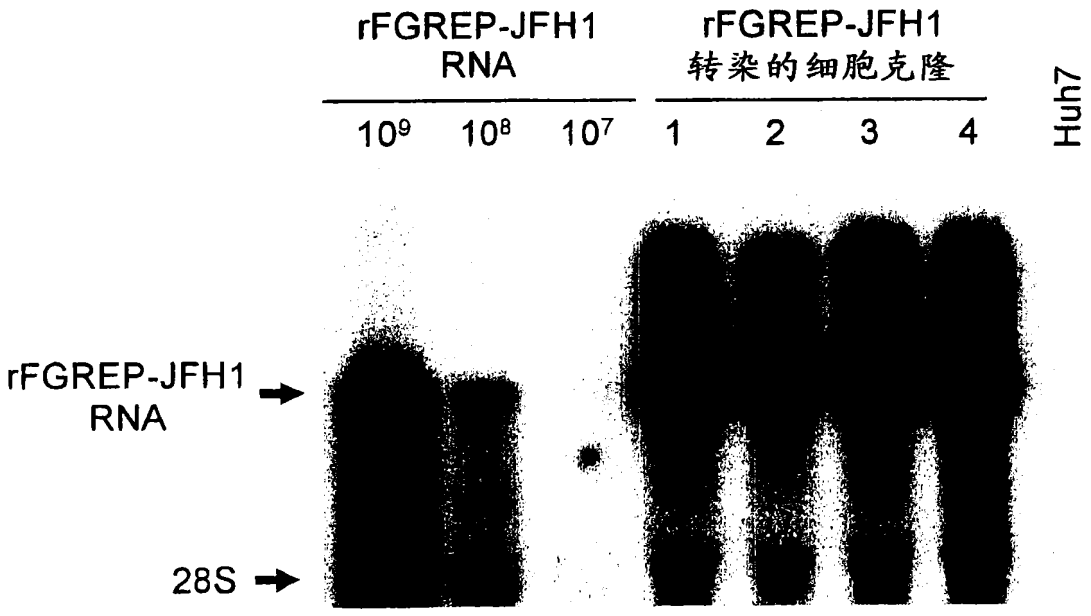

rFGREP-JFH1
RNA
rFGREP-JFH1
转染的细胞克隆
10⁹ 10⁸ 10⁷ 1 2 3 4
Huh7
rFGREP-JFH1
RNA
28S

图 7

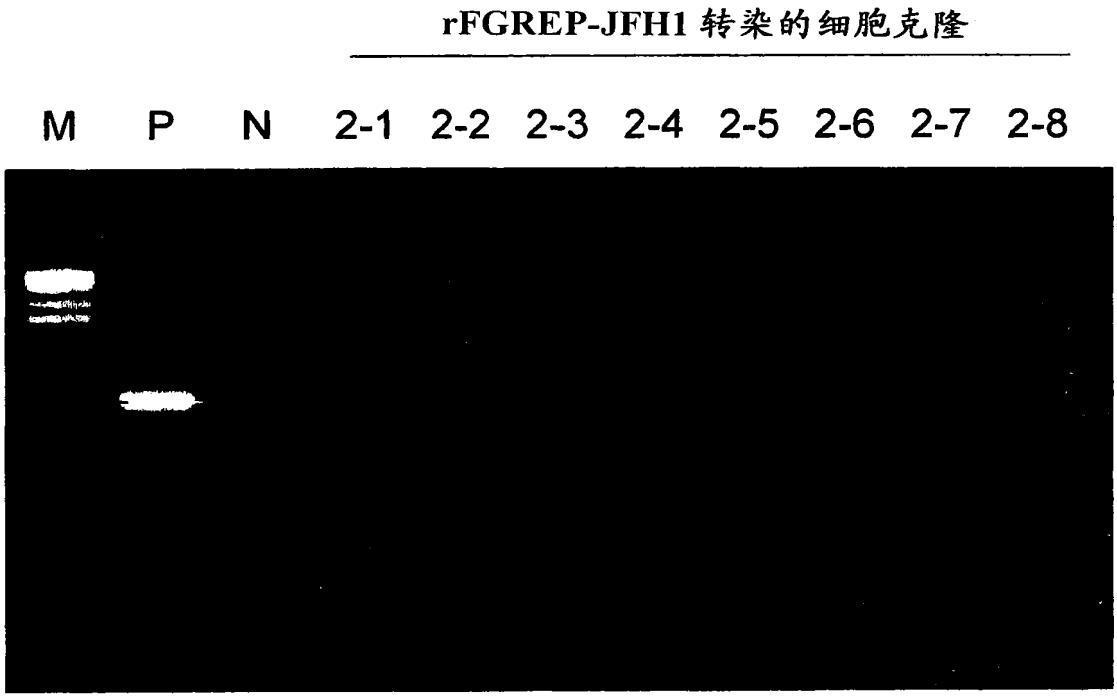


图 8

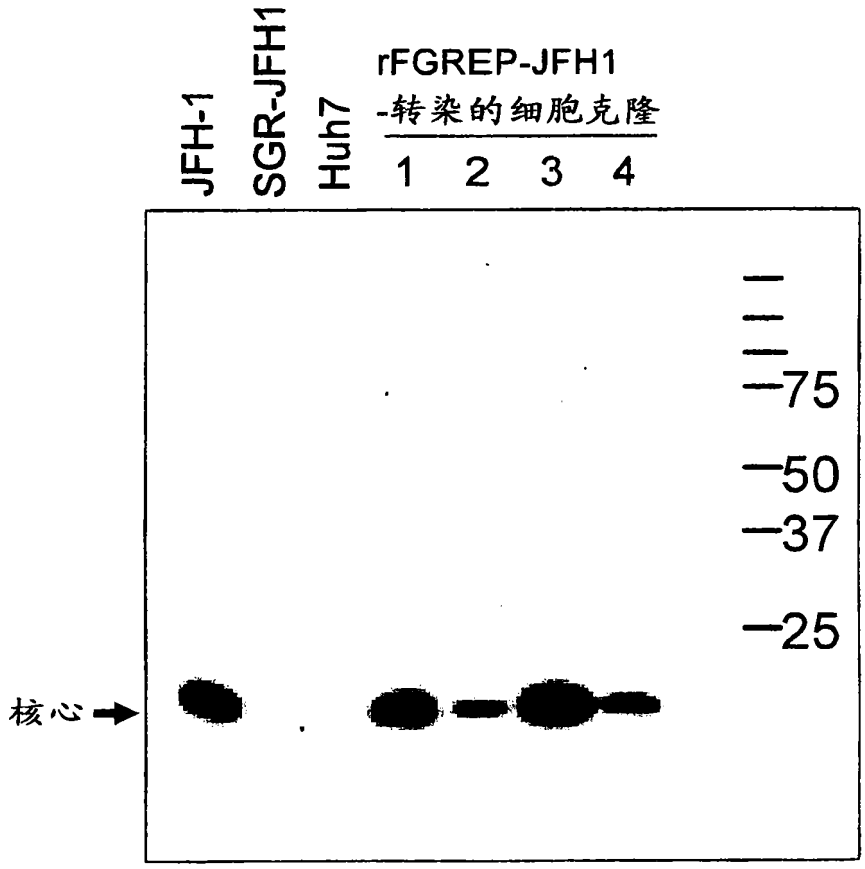


图 9

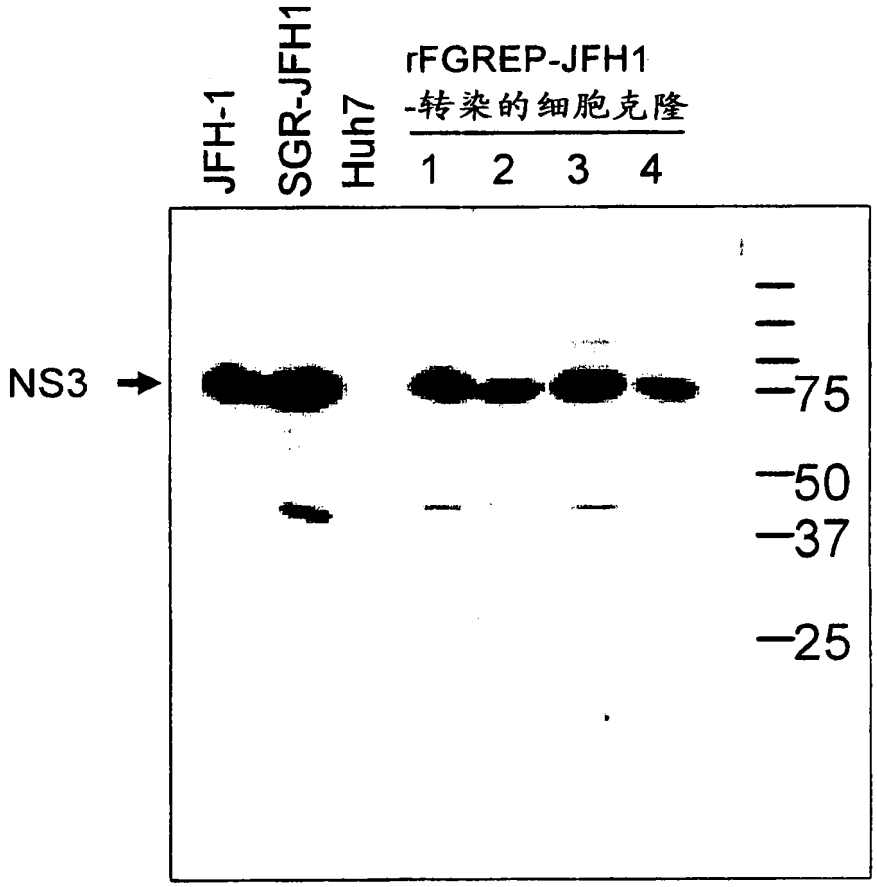


图 10

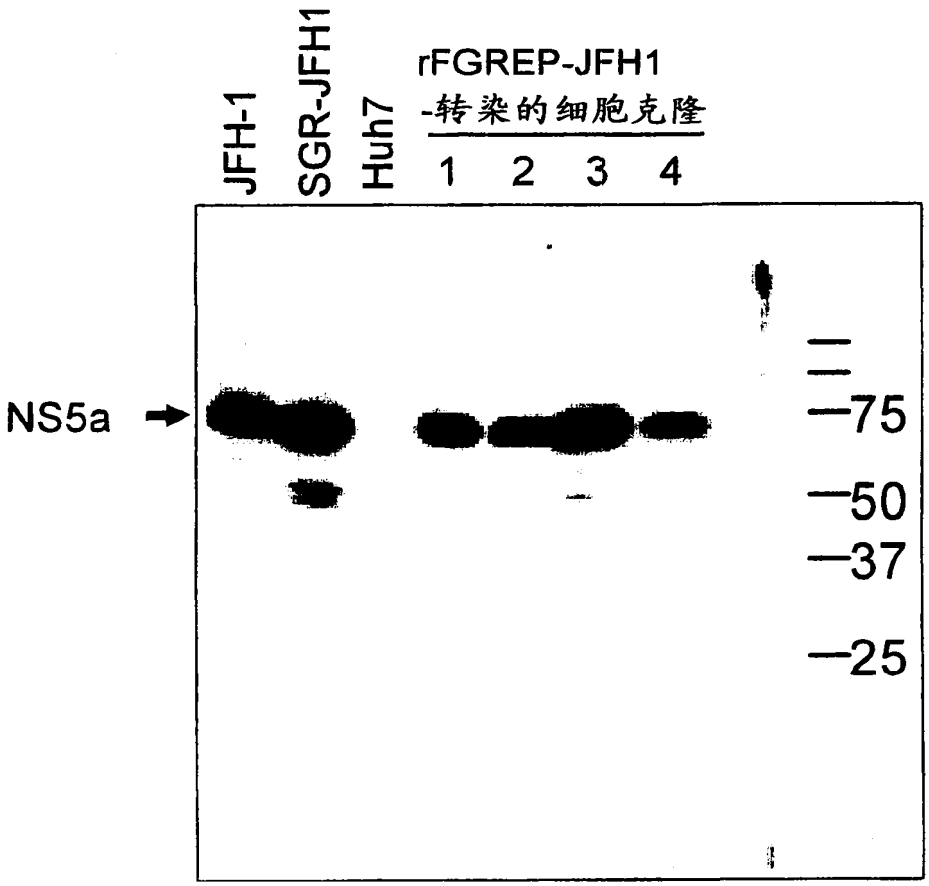


图 11

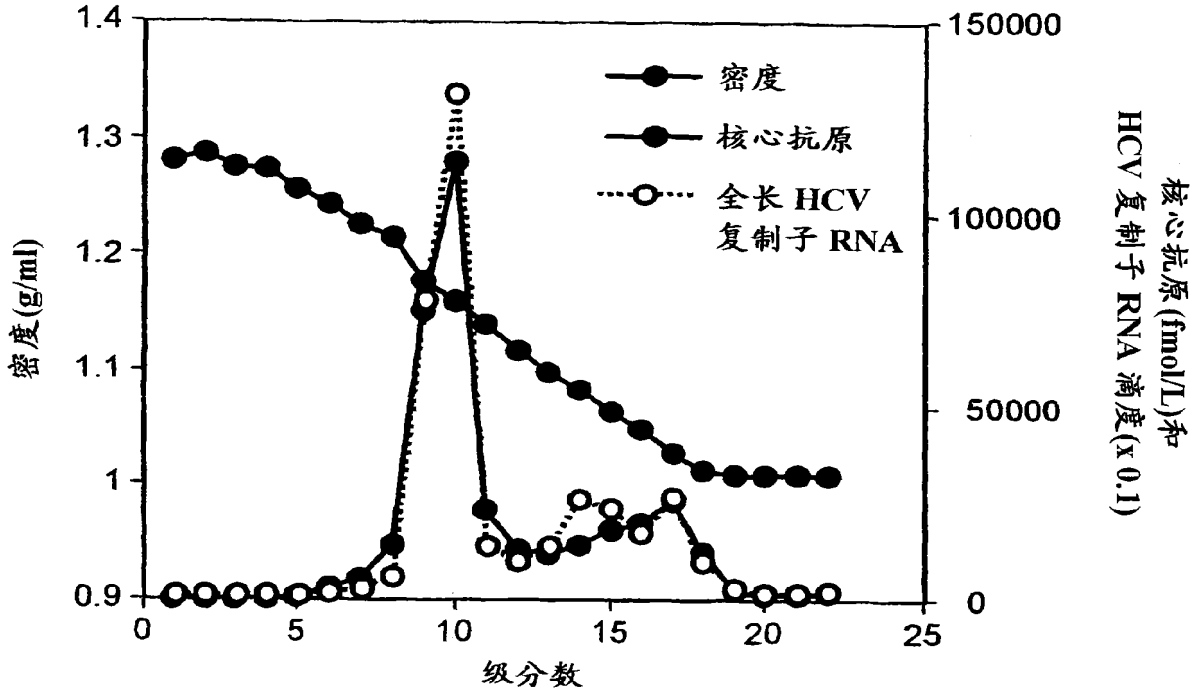
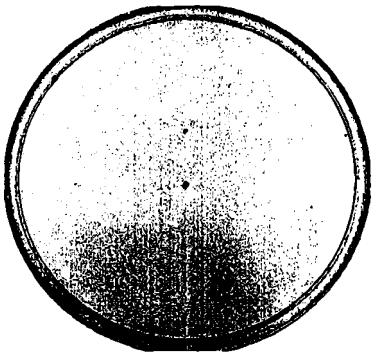
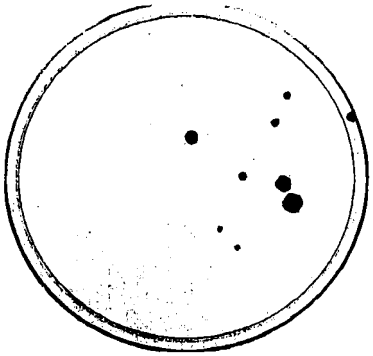

1.4
1.3
1.2
1.1
1
0.9
密度(g/ml)
0
5
10
15
20
25
级分数
150000
100000
50000
0
核心抗原(fmol/L)和
HCV 复制子 RNA 滴度(x 0.1)
密度
核心抗原
全长 HCV
复制子 RNA

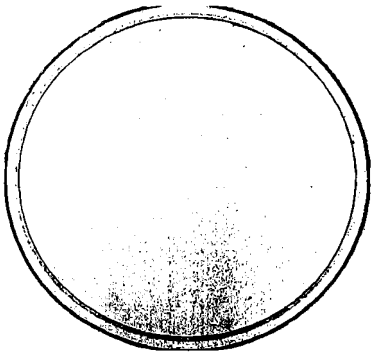
图 12



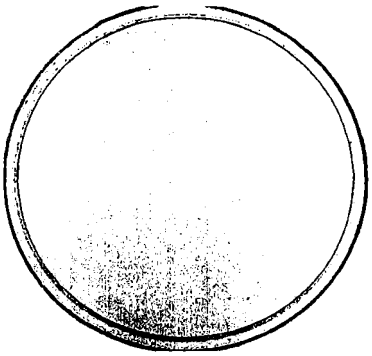
FGR-JFH1/2-3
4ml



FGR-JFH1/2-3
8ml



SGR-JFH1/4-1
4ml



SGR-JFH1/4-1
8ml