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CA 2813675 A1 2012/04/12
(21) **2 813 675**
(12) **DEMANDE DE BREVET CANADIEN**
CANADIAN PATENT APPLICATION
(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2011/10/07
(87) Date publication PCT/PCT Publication Date: 2012/04/12
(85) Entrée phase nationale/National Entry: 2013/04/04
(86) N° demande PCT/PCT Application No.: JP 2011/073189
(87) N° publication PCT/PCT Publication No.: 2012/046836
(30) Priorité/Priority: 2010/10/08 (JP2010-228152)

(51) Cl.Int./Int.Cl. *C12N 15/09* (2006.01), *C12N 1/15* (2006.01), *C12N 1/19* (2006.01), *C12N 1/21* (2006.01), *C12N 5/10* (2006.01), *C12N 7/00* (2006.01)
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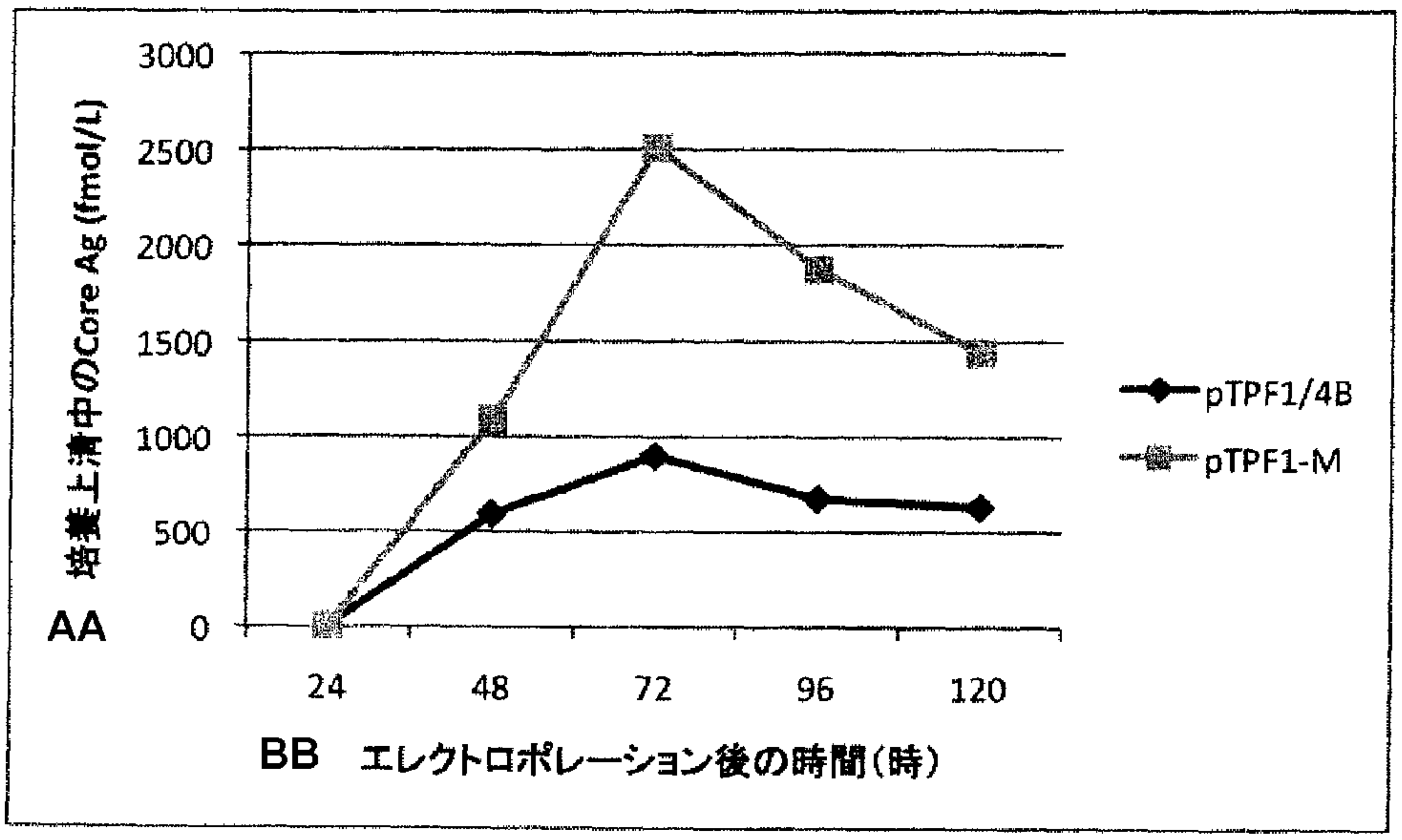
(54) Titre : GENE DU VIRUS DE L'HEPATITE C
(54) Title: HEPATITIS C VIRUS GENE

[図1]



AA Core Ag IN CULTURE SUPERNATANT (fmol/L)
BB TIME ELAPSED AFTER ELECTROPORATION (hour)

(57) Abrégé/Abstract:

Disclosed are: a hepatitis C virus (HCV) gene which has higher replication efficiency and higher reinfection efficiency than those of a known HCV gene having a genotype 1b; an RNA replicon which carries the gene; a cell which is infected by the RNA replicon and can replicate an HCV; and an HCV particle. The HCV gene is characterized in that the 979th amino acid encoded by the gene is threonine, the 1804th amino acid encoded by the gene is leucine and the 1966th amino acid encoded by the gene is lysine. It becomes possible to provide an HCV gene which can be proliferated in vitro and has higher replication efficiency and higher reinfection efficiency than those of a known HCV gene having a genotype 1b.

ABSTRACT

Disclosed are an HCV gene having higher replication efficiency and higher reinfection efficiency than the known HCV gene of genotype 1b, an RNA replicon having this gene, a cell infected with this RNA replicon, which cell allows replication of HCV, and an HCV particle. The hepatitis C virus gene encodes an amino acid sequence wherein the 979th amino acid is threonine; the 1804th amino acid is leucine; and the 1966th amino acid is lysine. An HCV gene which can propagate *in vitro* and has higher replication efficiency and higher reinfection efficiency than the known HCV gene of genotype 1b was provided.

DESCRIPTION

HEPATITIS C VIRUS GENE

TECHNICAL FIELD

[0001]

The present invention relates to a hepatitis C virus (which may be hereinafter referred to as "HCV") gene, an RNA replicon containing the gene, a cell infected with the RNA replicon, which cell allows replication of HCV, and an HCV particle.

BACKGROUND ART

[0002]

HCV is a causal factor of chronic hepatitis C, and, according to statistics reported by WHO, it is assumed that there are 170 million infected patients in the world. HCV is a virus classified into the genus *Flavivirus* in the family Flaviviridae. It is considered that infection with the virus occurs via blood or a blood component, and the virus grows in liver. In the initial phase of infection, patients infected with HCV show only mild symptoms, but the infection becomes chronic at high frequency, leading, after a certain length of asymptomatic period, to development of chronic hepatitis. As the infection continues, exacerbation of the disease condition occurs to cause liver cirrhosis, which then leads to liver cancer at high frequency. It is considered that hepatitis virus is involved in 95% of liver cancer, and that infection with HCV is responsible for 80% of such cases.

[0003]

For treatment of chronic hepatitis C, interferon is widely used. In recent years, the rate of complete cure by elimination of HCV *in vivo* has been gradually increasing due to improved formulations of interferon and improved administration methods such as combination therapy with interferon and ribavirin. However, the complete cure rate by administration of interferon is still about 50%, and it is considered that many types of HCV are resistant to interferon therapy. Thus, development of a drug having a therapeutic effect against the interferon-resistant virus has been demanded.

[0004]

Development of such a drug requires a drug screening system. Although a method by infecting cells derived from human or monkey with HCV *in vitro* and propagating HCV has been reported, such a propagation system could not be used as a drug screening system because of both low infection efficiency and low growth efficiency.

[0005]

Wakita et al. isolated an HCV gene of genotype 2a from a fulminant hepatitis C patient (Patent Document 1). From the isolated JFH1 strain, full-length RNA was synthesized *in vitro*, and the RNA was transfected into human liver cancer-derived cells (Huh7 cells). As a result, a replicon RNA that autonomously replicates in the cells was successfully obtained. Further, release of infectious particles into the culture supernatant of the cells into which the replicon RNA was transfected was confirmed (Non-patent Document 1). Therefore, by transfecting the replicon RNA of the JFH1 strain into human liver cancer-derived cells (Huh7 cells) and culturing the obtained infectious particles again with human liver cancer-derived cells, a reinfection-propagation system can be constructed. By using this reinfection-propagation system, screening of drugs against HCV has been started.

[0006]

However, the JFH1 strain is HCV of genotype 2a, which is an interferon-sensitive HCV. Therefore, since the strain does not have an HCV gene region responsible for resistance to interferon, host factors that act on the region providing resistance to interferon cannot be identified. Thus, screening of drugs effective against interferon-resistant HCV might be impossible.

[0007]

Lemon et al. reported an infection-propagation system prepared by transfecting the replicon RNA of the H77 strain of genotype 1a into human liver cancer-derived cells (Huh7 cells) (Non-patent Document 2). However, when virus particles obtained from the culture supernatant of the cells having the transfected replicon RNA were used again for infection of the human liver cancer-derived cells, the infectivity titer was 400 times lower than that of infectious particles of the JFH1 strain. Therefore, it is considered that the replicon RNA of the H77 strain releases virus particles which lost the infectivity. Thus, it is considered that the replicon RNA of the H77 strain which can replicate *in vitro* has already lost the function to produce infectious particles and does not have the inherent growth function of HCV. Thus, screening of drugs effective against HCV having the function to grow in the living body might be impossible with a screening system using the infection-propagation system of the replicon RNA of the H77 strain.

[0008]

Although, as described above, the replicon RNAs reported by Wakita and by Lemon partially enabled screening of drugs, those replicon RNAs have the above-described problems, and it is considered that screening of drugs that can be widely used for treatment of HCV is impossible with those replicon RNAs.

[0009]

Further, for obtaining a drug that can be widely used for treatment of HCV, the present inventors also developed pTPF1/4B as an *in vitro* propagation system which has an efficient HCV propagation, gene of genotype 1b, resistance to interferon, and ability to produce infectious particles (Patent Document 2). However, this culture system was practically problematic in Huh7 cells, which are human liver cancer-derived cells, since the efficiencies of autonomous replication in the cell, self-replication of RNA and secretion of virus particles into the culture supernatant were poor. Further, in Non-patent Document 3, it is reported that there is a region, in the C-terminus side of the NS2 region, which is important for infectivity.

PRIOR ART DOCUMENTS

[Patent Documents]

[0010]

[Patent Document 1] JP 2002-171978 A

[Patent Document 2] WO2008/136470

[0011]

[Non-patent Document 1] Nature Medicine, 2005, vol. 11, pp. 791-796.

[Non-patent Document 2] Proceeding of the National Academy of Science of the United State of America, 2006, vol. 103, pp. 2310-2315.

[Non-patent Document 3] Journal of Virology, 2009, vol. 83, pp. 12702-12713.

SUMMARY OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0012]

As described above, the present inventors discovered that TPF1/4B replicon RNA, which is an HCV gene derived from a fulminant hepatitis C patient showing resistance to interferon and has two amino acid mutations in the region of NS4B protein, shows high replication efficiency and releases infectious particles into the culture supernatant (Patent Document 2). However, for its application to a high-throughput screening system for more efficient drug search, an improved HCV gene having higher replication efficiency and higher reinfection efficiency needs to be obtained.

[0013]

Accordingly, the present invention aims to provide an HCV gene having higher replication efficiency and higher reinfection efficiency than the known HCV gene described in Patent Document 2. The present invention also aims to provide an RNA replicon having the gene of the present invention described above, a cell infected with the RNA replicon, which cell allows replication of HCV, and an HCV particle.

MEANS FOR SOLVING THE PROBLEMS

[0014]

As a result of intensive study, the present inventors discovered that, by mutating the 170th amino acid in the NS2 region in the gene of the TPF1/4B strain described in Patent Document 2 from Met to Thr, the replication efficiency and the reinfection efficiency can be significantly increased, thereby completing the present invention.

[0015]

That is, the present invention provides a hepatitis C virus gene that encodes threonine as the 979th amino acid, leucine as the 1804th amino acid and lysine as the 1966th amino acid. The present invention also provides an RNA replicon comprising the gene of the present invention. The present invention also provides a cell infected with the RNA replicon of the present invention, which cell allows replication of hepatitis C virus. The present invention also provides a hepatitis C virus particle having the gene of the present invention.

EFFECTS OF THE INVENTION

[0016]

By the present invention, an HCV gene which is capable of propagating *in vitro* and has higher replication efficiency and higher reinfection efficiency than the known HCV gene described in Patent Document 2 was provided for the first time. By using the HCV gene of the present invention, *in vitro* analysis of the HCV genome replicating *in vivo* is possible. By using this HCV genome, an infected-cell model simulating its replication in the liver of a patient that leads to exacerbation of hepatitis can be constructed. By using this model, development and screening of pharmaceutical agents that suppress/inhibit exacerbation of hepatitis are possible.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017]

Fig. 1 is a diagram showing the relationship between the time after electroporation by which the RNA replicon having the HCV gene of the present invention (pTPF1-M) prepared in Example below was transfected into cells and the concentration of the core antigen in the culture supernatant, together with the result for the HCV gene described in Patent Document 2 (pTPF1/4B).

Fig. 2 is a diagram showing the relationship between the time after electroporation by which the RNA replicon having the HCV gene of the present invention (pTPF1-M) prepared in Example below was transfected into cells and the concentration of the HCV RNA secreted into the culture supernatant, together with the result for the HCV gene described in Patent Document 2 (pTPF1/4B).

Fig. 3 is a diagram showing the relationship between the culture period after electroporation by which the RNA replicon having the HCV gene of the present invention (pTPF1-M) prepared in Example below was transfected into cells and the virus infectivity titer in the culture supernatant after the corresponding culture period, together with the result for the HCV gene described in Patent Document 2 (pTPF1/4B).

Fig. 4 is a diagram showing changes with time in the measured values of the core antigen in ALS32 cells and Huh7 cells after transfection of pTPF1-M RNA having the HCV gene of the present invention (pTPF1-M) prepared in Example below with an electroporator.

Fig. 5 is a diagram showing changes with time in the amounts of HCV RNA secreted into the culture supernatant of ALS32 cells and Huh7 cells after transfection of pTPF1-M RNA having the HCV gene of the present invention (pTPF1-M) prepared in Example below.

Fig. 6 is a diagram showing the result obtained by transfecting pTPF1-M RNA having the HCV gene of the present invention (pTPF1-M) prepared in Example below into each of ALS32 cells and Huh7 cells to allow secretion of virus particles formed from the HCV RNA into the culture supernatant, followed by infection of each of ALS32 cells and Huh7 cells with the virus particles. The relationship between the time period before harvest of the culture supernatant that was used for the infection and the virus infectivity titer is shown.

BEST MODE FOR CARRYING OUT THE INVENTION

[0018]

As described above, the HCV gene of the present invention is characterized in that the gene encodes threonine as the 979th amino acid, leucine as the 1804th amino acid and lysine as the 1966th amino acid. Among these, the leucine as the 1804th amino acid and the lysine as the 1966th amino acid are characteristics of the gene of the TPF1/4B strain described in Patent Document 2, and these two mutations allow self-replication of HCV *in vitro* and hence culture of HCV *in vitro*. The HCV of the present invention has these two mutations characteristic to the TPF1/4B strain, and additionally has a mutation in the NS2 region when compared to the gene of the TPF1/4B strain. That is, the amino acid sequence encoded by the HCV gene has threonine (instead of methionine in the TPF1/4B strain) at position 979 (170th amino acid in the NS2 region). SEQ ID NO:3 shows the nucleotide sequence of only the NS2 region together with the amino acid sequence encoded thereby, and SEQ ID NO:4 shows only the amino acid sequence.

[0019]

SEQ ID NO:1 shows the nucleotide sequence of the HCV gene obtained in Example below together with the amino acid sequence encoded thereby. SEQ ID

NO:2 shows only the amino acid sequence in SEQ ID NO:1. The above-described amino acid positions defined in the present invention are based on the amino acid sequence shown in SEQ ID NO:2, and, since the amino acid sequence shown in SEQ ID NO:2 has the same 3010 amino acids as those in the amino acid sequence of the common 1b-type HCV except for the amino acids at above-described sites having mutations, the positions are the same as those in the amino acid sequence of the common 1b-type HCV. In cases where the number of amino acids is not 3010, the positions of the above-described 3 amino acids defined in the present invention in the amino acid sequence can be easily identified by using well-known software for calculation of the sequence identity (mentioned later) to align the sequence with the amino acid sequence shown in SEQ ID NO:2 such that the number of matched amino acids is maximum. In such cases, the amino acids at the identified sites need to be the above-described 3 amino acids. In the nucleotide sequence shown in SEQ ID NO: 1 and other known 1b-type HCV genes, 5'-UTR corresponds to positions 1 to 341 in the nucleotide sequence; the core corresponds to positions 342 to 914 in the nucleotide sequence; the E1 region corresponds to positions 915 to 1490 in the nucleotide sequence; the E2 region corresponds to positions 1491 to 2579 in the nucleotide sequence; the P7 region corresponds to positions 2580 to 2768 in the nucleotide sequence; the NS2 region corresponds to positions 2769 to 3419 in the nucleotide sequence; the NS3 region corresponds to positions 3420 to 5312 in the nucleotide sequence; the NS4A region corresponds to positions 5313 to 5474 in the nucleotide sequence; the NS4B region corresponds to positions 5475 to 6257 in the nucleotide sequence; the NS5A region corresponds to positions 6258 to 7598 in the nucleotide sequence; the NS5B region corresponds to positions 7599 to 9371 in the nucleotide sequence; and 3'-untranslated region corresponds to positions 9372 to 9594. Therefore, among the mutation sites in the gene of the present invention, the 1804th amino acid corresponds to the 93rd amino acid in the NS4B region, and the

1966th amino acid corresponds to the 255th amino acid in the NS4B region.

[0020]

The HCV gene of the present invention includes not only those composed of RNA but also those composed of DNA. In cases of RNA, each "t" in the nucleotide sequences shown in SEQ ID NO:1 and SEQ ID NO:3 is read as "u" instead. Accordingly, within the scope of the present specification and claims, when a nucleotide sequence is mentioned, the nucleotide sequence shown by SEQ ID NO is interpreted to also include the nucleotide sequence having u instead of t.

[0021]

As is evident from the fact that various variants are known for the HCV gene, even a nucleotide sequence having a small number of mutations when compared with the nucleotide sequence shown in SEQ ID NO:1 can constitute hepatitis C virus having replication capacity and infection ability, and a gene which encodes an amino acid sequence having the above-described 3 amino acids and constituting hepatitis C virus having replication capacity and infection ability is also included within the scope of the present invention. That is, a gene which encodes an amino acid sequence having a sequence identity of not less than 95%, preferably not less than 99% with the amino acid sequence shown in SEQ ID NO:2 and constituting hepatitis C virus having replication capacity and infection ability (provided that the 979th amino acid is threonine; 1804th amino acid is leucine; and 1966th amino acid is lysine) is included within the scope of the present invention. The sequence identity herein means a value determined by aligning two amino acid sequences such that the number of matched amino acid residues is maximum (by inserting a gap(s) as required) and dividing the number of matched amino acid residues by the number of amino acid residues in the full-length sequence (in cases where the two sequences have different total numbers of amino acid residues, amino acid residues in the longer sequence). Such calculation of the sequence identity can be easily carried out using well-known software such as genetic analysis software including BLAST and MacVector (Version 10.5.1, MacVector). In cases where the gene is used for drug-discovery screening, the HCV is preferably as close to naturally occurring HCVs as possible. Therefore, mutations other than the 3 amino acid mutations characteristic to the present invention are preferably those found in naturally occurring HCVs. Further, the nucleotide sequence is preferably the nucleotide sequence shown in SEQ ID NO:1 (wherein t may instead be u) or a sequence having a sequence identity of not less than 90%, more preferably not less than 95%, still more preferably not less than 99% therewith. Whether or not the virus has replication capacity and infection ability can be judged by infecting a hepatic cell strain such as Huh7 cells with the virus and seeing if HCV can be detected in the culture supernatant of the cells, and a specific method is described in Example below.

[0022]

The HCV gene having the nucleotide sequence shown in SEQ ID NO:1 is the 1b type. Since the 1b type shows interferon resistance, it is important as a subject of drug-discovery screening. Thus, the HCV gene of the present invention is preferably the 1b type. Genotypes of HCV have been well studied, and methods of genotyping are described in literatures such as Okamoto et al. (Virology, 1992, vol. 188, pp. 331-341) and Simmonds et al. (Journal of General Virology, 1993, vol. 74, pp. 2391-2399). Those skilled in the art can easily carry out genotyping of HCV.

[0023]

The HCV gene of the present invention can be prepared by introducing a mutation(s) into a known HCV gene, preferably the known 1b-type HCV gene, by a method such as the well-known site-directed mutagenesis, such that the amino acid sequence encoded by the gene has the above-described 3 amino acids. Since, as described above, the TPF1/4B strain described in Patent Document 2 has the two mutations in the NS4B region, in cases where the TPF1/4B strain is used as the starting gene, the HCV gene of the present invention can be prepared by introducing one mutation into the NS2 region. Since the method of site-directed mutagenesis is well known and kits therefor are commercially available, this can be easily carried out. The introduction of mutation can be carried out by, for example, performing PCR (PCR Protocols, Academic Press (1990)) using specific primers to introduce the amino acid substitution to the NS2 region (see Example below). The gene may be cloned into, for example, pGEM-T easy vector (manufactured by Promega), and its nucleotide sequence may be determined with an automatic sequencer. Further, by digesting the HCV gene having the amino acid substitution with restriction enzymes *Age*I and *Fse*I and ligating the resulting fragment to pTPF1/4B similarly digested with the restriction enzymes, the full-length HCV gene of the present invention having the mutation in the NS2 region can be obtained (see Example below). In cases where another type of HCV gene is used as the starting gene, additional two mutations need to be introduced into the NS4B region, and the introduction can be easily carried out similarly by site-directed mutagenesis or the like. The HCV gene can be obtained by recovering HCV particles from blood of a hepatitis patient and extracting RNA by a conventional method.

[0024]

The replicon RNA (also called RNA replicon) of the present invention having the HCV gene of the present invention described above can be prepared using an arbitrary genetic engineering technique. The replicon RNA can be prepared by, for example, the following method, although the method of preparation is not restricted.

[0025]

The DNA encoding a replicon RNA is inserted into a cloning vector by a conventional method, to prepare a DNA clone. This DNA is inserted into the downstream of an RNA promoter, to prepare a DNA clone that can construct the replicon RNA. The RNA promoter is preferably contained in a plasmid clone.

Examples of the RNA promoter include, but are not limited to, T7 RNA promoter, SP6 RNA promoter and SP3 RNA promoter, and the RNA promoter is especially preferably T7 RNA promoter.

[0026]

The vector into which the DNA is to be inserted is not restricted, and examples of the vector include plasmid vectors; linear double-stranded DNA vectors; and virus vectors such as adenovirus vectors, adeno-associated virus vectors, retrovirus vectors and lentivirus vectors. The vector is preferably a plasmid vector.

[0027]

The replicon RNA of the present invention can be prepared from the vector into which the DNA was inserted. Using the DNA clone as a template, RNA is synthesized with RNA polymerase. The RNA synthesis can be started from the 5'-untranslated region by a conventional method. In cases where the template DNA is a plasmid clone, the DNA region linked to the downstream of an RNA promoter may be cleaved out with a restriction enzyme(s), and the obtained DNA fragment may be used as a template to synthesize RNA. The 3'-end of the synthesized RNA is preferably the same as the 3'-untranslated region of the virus genome RNA, with neither addition nor deletion of another sequence thereto. For example, in a preferred mode of the full-length replicon RNA of the present invention, the DNA is inserted into a vector having a T7 RNA promoter upstream of the 5'-UTR and a *Xba*I restriction site at the end of 3'-UTR. The resulting vector is digested with *Xba*I, and the HCV genomic RNA can then be synthesized with T7 RNA polymerase.

[0028]

The replicon-replicating cell of the present invention can be prepared by transfecting the RNA replicon into an arbitrary cell. The cell into which the replicon RNA is to be transfected is not restricted, and the cell is preferably a human liver-derived cell, mouse liver-derived cell or monkey liver-derived cell, especially preferably Huh7 cell, HepG2 cell or Hep3B cell, which are human liver cancer-derived cells, or IMY-N9 cell, HeLa cell, CHO cell, COS cell, Vero cell or 293 cell. The human liver cancer-derived cells Huh7 cell, HepG2 cell and Hep3B cell are especially preferred. By subjecting these cells, preferably human liver cancer-derived cells, to limiting dilution to obtain monoclonal cells, and transfecting the replicon RNA to the obtained cells, a large amount of the replicon RNA can be produced, which is preferred. For example, as concretely described in Example below, when the RNA replicon was transfected into ALS32 cells, which are monoclonal cells established by subjecting human liver cancer-derived Huh7 cells to limiting dilution (medium: D-MEM medium supplemented with 10% FBS), a much larger amount of the replicon RNA was produced than in the case where the RNA replicon was transfected into their parent cells, Huh7 cells. The replicon RNA can be transfected into the cells by an arbitrary transfection method. Examples of such a transfection method include electroporation, particle gun method and lipofection. Among these, the method by electroporation is especially preferred.

[0029]

In cases where a replicon RNA containing a selection marker gene or reporter gene is used in the transfection into cells, cells into which the replicon RNA was transfected and in which the replicon RNA is continuously replicating can be selected utilizing expression of the selection marker gene or reporter gene. For example, in cases where the replicon RNA contains a neomycin-resistance gene as a selection marker gene, the cells after transfection with the replicon RNA are plated in a culture dish, and G418 (neomycin) is added thereto at a concentration of 0.05 mg/ml to 3.0 mg/ml. Thereafter, the culture is continued while the culture medium is replaced twice a week. The cells become visible as colonies 2 or 3 weeks after the plating.

[0030]

The replicon-replicating cell of the present invention produces replicon RNA, hepatitis C virus protein and hepatitis C virus particles. Therefore, the replicon-replicating cell can be used for producing replicon RNA, hepatitis C virus protein and hepatitis C virus particles.

[0031]

The replicon RNA replicated in the replicon-replicating cell can be extracted from the cell by an arbitrary RNA extraction method. The RNA extracted from the cell can be made to function as the replicon RNA again by transfection into the cell. The cell which may be used in this case is Huh7 cell, HepG2 cell or Hep3B cell, which are human liver cancer-derived cells, or IMY-N9 cell, HeLa cell, CHO cell, COS cell, Vero cell or 293 cell; more preferably Huh7 cell, HepG2 cell or Hep3B cell, which are human liver cancer-derived cell; especially preferably a monoclonal cell obtained by subjecting these human liver cancer-derived cells to limiting dilution, which monoclonal cell has improved replication capacity of the replicon RNA compared to its parent cell (e.g., ALS32 cell described in Example). As the hepatitis C virus protein of the present invention, either the protein in the cell or the protein secreted into the culture supernatant may be used. The produced hepatitis C virus protein can be extracted and purified by known methods. As the hepatitis C virus particles produced by the replicon-replicating cell, either the particles in the cell or the particles secreted into the culture supernatant may be used. The replicon RNA may be altered to modify the hepatitis C virus protein and hepatitis C virus particles of the present invention, in order to reduce the pathogenicity by modification of the RNA, virus protein or virus particles for use as a vaccine.

[0032]

By using the replicon-replicating cell, screening of substances that control infection with hepatitis C virus can be carried out. The term “control infection with hepatitis C virus” means, for example, regulation (e.g., promotion or suppression) of replication of HCV RNA or regulation (e.g., promotion or suppression) of translation from RNA into protein.

[0033]

More specifically, screening of a test substance can be carried out by bringing the test substance into contact with the replicon-replicating cells and analyzing the degree of increase in the replicon RNA. The degree of increase in the replicon RNA means the amount of change in the replication rate or in the amount of replicon RNA. More specifically, screening of a test substance can be carried out by detecting or measuring the amount of replicon RNA in the replicon cells or in the supernatant, and comparing the measured value with the amount of replicon RNA in control replicon-replicating cells that were not brought into contact with the test substance. The screening of a test substance can also be carried out by detecting or measuring the amount of hepatitis C virus protein in the cells or in the supernatant, and comparing the measured value with that of replicon-replicating cells that were not brought into contact with the test substance. The hepatitis C virus protein which can be detected or measured in the screening is not restricted, and the protein is preferably the core protein. The core protein may also be measured using a commercially available kit. Further, automation of the screening method may be applied to a high-throughput screening process.

[0034]

Further, the screening method of the present invention is also effective as a method for evaluation of an effect of a screened drug. In cases where evaluation of a drug needs to be carried out by this screening method, the method can also be used as a method for producing the drug.

[0035]

The present invention is concretely described below by way of Example. However, the present invention is not limited to the Example below.

[0036]

REFERENCE EXAMPLE

Obtaining TPF1/4B Strain Gene

By the method described in Examples in Patent Document 2, the TPF1/4B strain gene was obtained and analyzed. That is, the following operation was carried out.

[0037]

(A) Extraction of RNA from Serum

From 250 μL of serum collected from a fulminant hepatitis patient in the acute stage, RNA was purified using the High Pure Viral Nucleic Acid Kit (Roche diagnostics corporation) according to the method recommended by the manufacturer.

[0038]

(B) Synthesis of cDNA, and Amplification of cDNA by PCR

To the purified RNA, the XR58R primer was added, and reverse transcription was performed with SuperSucript II reverse transcriptase (Invitrogen) according to the method recommended by the manufacturer at 42°C for 1 hour, to obtain cDNA. To the obtained reaction solution, RNase H (Invitrogen) was added, and the reaction was allowed to proceed at 37°C for 30 minutes to degrade RNA. The resulting reaction solution was subjected to polymerase chain reaction (PCR) using the HC-LongA1 primer, 1b9405R primer and Takara LA Taq DNA polymerase (Takara Shuzo Co., Ltd.) wherein thermal cycling reaction was performed by 30 cycles of 94°C for 20 seconds and 68°C for 9 minutes, to amplify the cDNA. Further, a part of the obtained reaction solution was subjected to PCR using the HC85F and HC9302R primers, to amplify HCV cDNA.

[0039]

(C) Cloning of cDNA

The amplified DNA fragment was separated by electrophoresis using 0.7% agarose gel, and recovered using the QIAquick gel purification kit (QIAGEN) according to the method recommended by the manufacturer. The recovered DNA fragment was subjected to ligation reaction with pGEM-T easy vector (Promega), and the resulting plasmid was used for transformation of the DH5α strain. An ampicillin-resistant transformant was selected and cultured using 2YT medium. From the cultured bacterial cells, plasmid was purified using Wizard Plus SV Miniprep DNA Purification System.

[0040]

(D) Determination of Nucleotide Sequence

The nucleotide sequence of HCV cDNA was determined using primers (SEQ ID NOs:17 to 39) designed based on the nucleotide sequence of HCV of genotype 1b. Using the CEQ DTCS Quick Start Kit (Beckman Coulter) according to the method recommended by the manufacturer, the reaction was performed, and analysis was carried out by the CEQ2000 XL DNA analysis system (Software version 4.0.0, Beckman Coulter). The obtained data were analyzed with Sequencher (Version 4.1.2, Gene Codes Corporation). The obtained HCV clone was designated pTPF1-0193.

[0041]

(E) Obtaining cDNA of 5'-Untranslated Region and Determining Its Nucleotide Sequence

Further, the RNA obtained in the above Step (A) was subjected to 5'-RACE to obtain cDNA at the end of the 5'-untranslated region. The reaction was performed using 5'-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen) according to the instruction provided by the manufacturer.

[0042]

As an antisense primer for cDNA synthesis, Chiba-as was used. cDNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen) and purified with S.N.A.P column, followed by subjecting the purified cDNA to TdT-tailing reaction to add dCTPs. Using the 5'RACE Abridged Anchor primer attached to the kit and KY78 primer, and Takara LA Taq DNA polymerase (Takara Shuzo Co., Ltd.), the first PCR was carried out. Using a part of the PCR product as a template, and the UTP primer attached to the kit and the KM2 primer, the second PCR was performed with Takara LA Taq DNA polymerase (Takara Shuzo Co., Ltd.) to obtain a PCR product. This PCR product was cloned into pGEM-T easy vector, and the nucleotide sequence was determined according to the above Step (D). The obtained HCV cDNA clone containing positions 1 to 709 in SEQ ID NO:1 was designated pTPF1-0007.

[0043]

(F) Obtaining cDNA of 3'-Untranslated Region and Determining Its Nucleotide Sequence

The RNA obtained in the above Step (A) was subjected to 3'-RACE to obtain cDNA at the end of the 3'-untranslated region. First, Poly(A) was added to the RNA of the patient using the Poly(A) Tailing Kit (Ambion) according to the instruction attached to the kit. The same operations as in the Steps (B) to (D) described above were carried out except that the dT-Adp primer was used instead of the XR58R primer; the 3UTR-1F primer and the Adp primer were used as the primers for the 1st PCR; and the XR58F and Adp primers were used as the primers for the 2nd PCR. The obtained HCV cDNA clone was designated pTPF1-8994.

[0044]

The obtained HCV strain was designated the TPF1 stain. The TPF1 strain is an HCV having a total length of 9594 bases. Its nucleotide sequence is shown in SEQ ID NO:1 (wherein C at position 3277 is read as T; T at position 5752 is read as A; and A at position 6237 is read as G). The polynucleotide of the obtained TPF1 strain had a coding region encoding 3010 continuous amino acids between position 342 and position 9374. The amino acid sequence of the polyprotein of the TPF1 strain is shown in SEQ ID NO:2 (wherein the amino acid T (threonine) at amino acid position 979 is read as M (methionine); the amino acid L (leucine) at amino acid position 1804 is read as Q (glutamine); and the amino acid K (lysine) at amino acid position 1966 is read as E (glutamic acid).

[0045]

The following are primers used for the cloning and the determination of nucleotide sequences.

XR58R (SEQ ID NO:5): 5'-tcatgcggct cacggacctt tcacagctag-3'
HC-LongA1 (SEQ ID NO:6): 5'-atcgtcttca cgcagaaagc gtctagccat-3'
1b9405R (SEQ ID NO:7): 5'-gcctattggc ctggagtgtt tagctc-3'
HC85F (SEQ ID NO:8): 5'-atggcgttag tatgagtgtc gtgcagcct-3'
HC9302R (SEQ ID NO:9): 5'-tcgggcacga gacaggctgt gatatatgtc t-3'
Chiba-as (SEQ ID NO:10): 5'-tgcacggtct acgagacct-3'
KY78 (SEQ ID NO:11): 5'-ctcgcaagca ccctatcagc cagt-3'
KM2 (SEQ ID NO:12): 5'-aggcattgag cgggtttat-3'
dT-Adp (SEQ ID NO:13): 5'-ctagactcga gtcgacatcg tttttttttt ttttttt-3'
3UTR-1F (SEQ ID NO:14): 5'-atcttagccc tagtcacggc-3'
Adp (SEQ ID NO:15): 5'-ctagactcga gtcgacatcg-3'
XR58F (SEQ ID NO:16): 5'-ctagctgtaa aggtccgtga gccgcatga-3'
M13 Primer M3 (SEQ ID NO:17): 5'-gtaaaacgac ggccagt-3'
M13 Primer RV (SEQ ID NO:18): 5'-caggaaacag ctatgac-3'
104 (SEQ ID NO:19): 5'-aggaagactt ccgagcggtc-3'
HC841S (SEQ ID NO:20): 5'-ggaacttgcc cggttgctct ttctctatct tc-3'
E1 (SEQ ID NO:21): 5'-attccatggt ggggaactgg gctaa-3'
HC2069S (SEQ ID NO:22): 5'-taacaatacc ttgacctgcc ccacggactg-3'
HC2430S (SEQ ID NO:23): 5'-aacatcgtgg acgtgcaata cctgtacgg-3'
HC2461AS (SEQ ID NO:24): 5'-gaccctacac cgtacaggta-3'

HC2769S (SEQ ID NO:25): 5'-ttggaccggg agatggctgc atcgtg-3'
HC3632F (SEQ ID NO:26): 5'-cacccaaatg tacaccaatg t-3'
HC3928S (SEQ ID NO:27): 5'-tacccgttga gtctatggaa ac-3'
HC4016AS (SEQ ID NO:28): 5'-cacttggaat gtctgcggta-3'
HC4498S (SEQ ID NO:29): 5'-agggggggag gcatctcatt ttctg-3'
HC4888F (SEQ ID NO:30): 5'-tgctatgacg cgggctgtgc ttggta-3'
HC5381F (SEQ ID NO:31): 5'-ggtcattgtg ggcaggatca t-3'
HC5692S (SEQ ID NO:32): 5'-ctgcctggaa accccgcgat-3'
HC5858F (SEQ ID NO:33): 5'-tggcagcata ggccttggga aggt-3'
HC6315F (SEQ ID NO:34): 5'-aagacctggc tccagtccaa g-3'
5A-1 (SEQ ID NO:35): 5'-ttccatgctc accgaccccct c-3'
HC7090S (SEQ ID NO:36): 5'-gtggagtcag agaataaggt-3'
HC7743F (SEQ ID NO:37): 5'-cagaagaagg tcacctttga c-3'
HC8192S (SEQ ID NO:38): 5'-gcagcgggtc gagttcctgg tgaat-3'
HC8939F (SEQ ID NO:39): 5'-ctacggggcc tgttactcca ttgaac-3'

[0046]

(G) Preparation of Subgenomic RNA Replicon

The full-length polynucleotide of the hepatitis C virus TPF1 strain was inserted into the downstream of the T7 RNA promoter sequence in pBluescript II SK(+) (hereinafter referred to as pTPF1).

[0047]

Thereafter, in pTPF1, a region from a part of the core region to the NS2 region, encoding structural protein and nonstructural protein, was replaced with a neomycin resistance gene (neomycin phosphotransferase, NPT-II) and EMCV-IRES (internal ribosomal entry site of encephalomyocarditis virus) to construct a plasmid DNA pRepTPF1. This plasmid was constructed by a procedure according to a previous report (Lohmann et al., Science, (1999) 285, pp.110-113).

[0048]

More specifically, pTPF1 was first digested with restriction enzymes *Age*I and *Bsr*GI. A fragment obtained by amplifying a sequence from the 5'-UTR to the core region derived from pTPF1 and a neomycin resistance gene derived from pcDNA3.1(+) by PCR and digesting the resulting amplification product with restriction enzymes *Age*I and *Pme*I; and a fragment obtained by linking a sequence from EMCV-IRES to the NS3 region by PCR amplification and digesting the resulting product with restriction enzymes *Pme*I and *Bsr*GI; were inserted into the above *Age*I/*Bsr*GI-digested pTPF1 by ligation, to obtain a plasmid DNA pRepTPF1.

[0049]

Into this plasmid DNA pRepTPF1, mutations were introduced using the Quick Mutagenesis Kit (Stratagene) according to the method recommended by the manufacturer such that the nucleic acid corresponding to nucleotide position 5752 in SEQ ID NO:1 is substituted from A to T and the nucleic acid corresponding to nucleotide position 6237 is substituted from G to A. As a result, the amino acid corresponding to amino acid position 1804 in SEQ ID NO:2 was mutated from Q (glutamine) to L (leucine), and the amino acid corresponding to amino acid position 1966 was mutated from E (glutamic acid) to K (lysine). The plasmid DNA into which these amino acid substitutions were introduced was designated pRep4B.

[0050]

The plasmid DNA pTPF1 containing the full-length HCV DNA prepared in (F) was digested with a restriction enzyme *Sfi*I, and a fragment obtained by digesting pRep4B with a restriction enzyme *Sfi*I was inserted into the above cleavage site by ligation, to prepare a plasmid DNA pTPF1/4B containing the full-length HCV DNA into which the appropriate mutations were inserted. The thus obtained HCV strain was designated the TPF1/4B strain.

[0051]

EXAMPLES

1. Introduction of Mutation into HCV NS2 Protease Region

Using the pTPF1/4B gene as a template, in the presence of the AgeI primer 5'-accggtgagtacaccggaattgccaggacg-3' (SEQ ID NO:40) and the FseI primer 5'-atttgggtgattgggcccttcgggccggcc-3' (SEQ ID NO:41), polymerase chain reaction (PCR) was performed using Takara LA Taq DNA polymerase (Takara Shuzo Co., Ltd.) by thermal cycling reaction by 20 cycles of 94°C for 20 seconds and 68°C for 4 minutes, to amplify the vicinity of the NS2 protease region in the TPF1/4B genome.

[0052]

The amplified fragment was separated by 1.0% agarose gel electrophoresis, and the DNA fragment was recovered using the QIAquick gel purification kit (QIAGEN) according to the method recommended by the manufacturer. The recovered TPF1 fragment was ligated to pGEM-T easy vector (Promega) according to the method recommended by the manufacturer, and the DH5α strain was transformed with the obtained plasmid. A transformant which was resistant to ampicillin and formed a white colony in plate culture on agar medium supplemented with IPTG and X-gal was selected, and the selected transformant was cultured in 2YT medium supplemented with 100 μg/ml ampicillin. From the cultured bacterial cells, a plasmid pTPF1-AgeFse was purified using Wizard Plus SV Miniprep DNA Purification System.

[0053]

The sequence of the TPF1 fragment incorporated in the purified plasmid was analyzed by performing reaction using the CEQ DTCS Quick Start Kit (Beckman Coulter) according to the method recommended by the manufacturer, with primers provided as appropriate that matches the vector and the HCV sequence, followed by analysis by the CEQ2000 XL DNA analysis system (Software version 4.0.0, Beckman Coulter). The obtained data was processed by Sequencher (Version 4.1.2, Gene Codes Corporation) for integration and analysis of the sequence data to confirm the nucleotide sequence of pTPF1-AgeFse.

[0054]

Thereafter, the mutation at amino acid position 170 (from M to T) in NS2 protease was introduced into the pTPF1-AgeFse prepared as described above, using the Quick Mutagenesis kit (Stratagene) according to the method recommended by the manufacturer. The plasmid into which the amino acid substitution was introduced was designated pTPF1-AgeFse/Npro.

[0055]

Further, pTPF1/4B was digested with restriction enzymes *Age*I and *Fse*I, and the fragment derived from pTPF1-AgeFse/Npro prepared by digestion with *Age*I and *Fse*I was inserted into the above cleavage site by ligation. This plasmid was designated pTPF1-M.

[0056]

2. Effect of Amino Acid Mutations on HCV RNA Replication

Using, as a template, each of pTPF1-M prepared in 1, which has the amino acid mutation, and pTPF1/4B, which does not have the sequence causing the mutation, RNA was synthesized with the Megascript T7 kit (Ambion) or AmpliScribe T7-Flash transcription kit (Epicentre). The RNA was purified according to the method recommended by the manufacturer.

[0057]

Using Dulbecco's modified Eagle medium (D-MEM, IWAKI) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin and 50 µg/mL streptomycin as a medium, human liver cancer cells (Huh7, JCRB0403) were cultured under 5% carbon dioxide at 37°C. Cells before confluence were detached from the culture dish by treatment with trypsin and EDTA, and resuspended in a serum-containing medium to inactivate trypsin. The cells were washed twice with PBS, and resuspended in Cytomix (120 mM potassium chloride, 10 mM potassium phosphate, 5 mM magnesium chloride, 25 mM HEPES, 0.15 mM calcium chloride and 2 mM EGTA, pH7.6) supplemented with 1.25% DMSO. The resulting cell suspension was then transferred to an electroporation cuvette with a gap of 0.4 cm.

[0058]

After adding 10 μg of RNA to the cells, the resulting mixture was sufficiently cooled on ice for 5 minutes. Using an electroporator (Bio-Rad), a pulse was applied to the cells at 960 μF at 250 V. The cells after transfection were immediately resuspended in 10 mL of a medium, and 1 mL each of the resulting suspension was placed in a 12-well plate (diameter, 22.1 mm), followed by starting culture. The culture supernatant was harvested at Hour 4, Hour 24, Hour 48 and Hour 72. After centrifuging the harvested culture supernatant at 2 k rpm for 10 minutes, the supernatant was recovered. Using a kit for the HCV core antigen (Fujirebio, Inc., LUMIPULSE), 100 μL of the supernatant was subjected to measurement.

[0059]

As shown in Fig. 1, the measurement values of the core antigen in pTPF1-M and pTPF1/4B after transfection of the RNA into the cells using an electroporator were highest at Hour 72 after the electroporation, and the amount of the core antigen of pTPF1-M secreted was 2.8 times higher than that of pTPF1/4B. Further, the amount of HCV RNA secreted into the culture supernatant was highest at Hour 72 after the electroporation as in the case of the core antigen, and the amount of the HCV RNA in pTPF1-M was 3 times higher than that of pTPF1/4B (Fig. 2). This indicates that the pTPF1-M gene of the present invention having the mutation introduced in the NS2 protease region replicated in the cells and secreted the core antigen and the HCV RNA into the supernatant more efficiently than the pTPF1/4B gene. This means that the replication efficiency of the pTPF1-M gene is higher than that of pTPF1/4B.

[0060]

3. Infection of Cultured Cells with HCV Particles

Whether or not the core antigen and the HCV RNA secreted into the culture supernatant in 2 form virus particles to allow reinfection *in vitro* was studied. More specifically, Huh7 cells were transfected with full-length HCV RNA synthesized from each of the pTPF1-M and pTPF1/4B genes, and the culture supernatant was harvested with time. The harvested culture supernatant was centrifuged at 15,000 rpm for 10 minutes and filtered (0.45μm, Millipore) to remove cell debris and the like.

[0061]

The supernatant after filtration was reacted for 6 hours at 37°C with naive Huh7 cells cultured in a 12-well plate (diameter, 22.1 μm). Thereafter, the cells were washed 3 times with PBS. A fresh growth medium was added to the cells, and the cells were cultured in an incubator under 5% carbon dioxide at 37°C. For evaluation of the virus infectivity titer, the core antigen accumulated in the cells was visualized by immunostaining, and the number of cells positive for the core antigen was counted, to represent the number of infective virus particles contained in the culture supernatant with the focus-forming unit (FFU/mL).

[0062]

More specifically, the cells 96 hours after the infection were fixed with methanol, and the fixed cells were incubated using 2% BSA-PBS for 2 hours at room temperature to perform blocking. Subsequently, an anti-core monoclonal antibody (1 μg/mL) as a primary antibody was added thereto, and the resulting mixture was incubated for 1 hour at room temperature. The cells were sufficiently washed, and an FITC-labeled secondary antibody was added thereto, followed by incubation of the resulting mixture for 1 hour at room temperature. HCV-infected cells were observed by counting the number of cells positive for the core antigen in the cells using an inverted fluorescence microscope.

[0063]

As shown in Fig. 3, in pTPF1-M having the mutation introduced in the NS2 protease region, the number of infectious virus particles released into the culture supernatant was largest (300 FFU/mL) at Hour 48 after the transfection of HCV RNA. On the other hand, in pTPF1/4B not having the amino acid mutation, although the number of infectious virus particles released into the culture supernatant was largest at Hour 48 (75 FFU/mL) after the transfection of HCV RNA, the value was as low as one-fourth of the value in pTPF1-M. Thus, it was shown that the cells into which the pTPF1-M gene was transfected released a larger number of infectious virus particles than the cells into which the pTPF1/4B gene was transfected. This indicates that the mutation in the NS2 protease region of the present invention improves the growth efficiency and the infection efficiency of the virus RNA.

[0064]

4. Propagation of Virus in Monoclonal Huh7 Cells

Human liver cancer cells (Huh7, JCRB0403) were subjected to limiting dilution to obtain monoclonal cells, to newly establish the ALS32 cell. The propagation performance of pTPF1-M in ALS32 cells was evaluated by electroporation.

[0065]

The human liver cancer cells (Huh7, JCRB0403) were cultured using, as a culture medium, D-MEM supplemented with 10% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin, under 5% carbon dioxide at 37°C. The cells before confluence were detached from the culture dish by treatment with trypsin and EDTA, and resuspended in a serum-containing medium to inactivate trypsin. The number of cells was measured, and the cells were plated on a 96-well plate after adjusting its density using a growth medium such that 1 cell is contained per 1 well. After the plating, the culture was continued for 1 month, to establish the ALS32 cell.

[0066]

Each of ALS32 cells and Huh7/JCRB0403 cells were cultured using, as a culture medium, D-MEM supplemented with 10% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin, under 5% carbon dioxide at 37°C. The cells before confluence were detached from the culture dish by treatment with trypsin and EDTA, and resuspended in a serum-containing medium to inactivate trypsin. The cells were washed twice with PBS, and resuspended in Cytomix supplemented with 1.25% DMSO. The resulting cell suspension was then transferred to an electroporation cuvette with a gap of 0.4 cm.

[0067]

To each of ALS32 cells and Huh7/JCRB0403 cells, 10 μg of the pTPF1-M RNA synthesized in Example 3 was added, and the resulting mixture was sufficiently cooled on ice for 5 minutes. Using an electroporator (Bio-Rad), a pulse was applied to the cells at 960 μF at 250 V. The cells after transfection were immediately resuspended in 10 mL of a medium, and 1 mL each of the resulting suspension was placed in a 12-well plate (diameter, 22.1 mm), followed by starting culture. The culture supernatant was harvested at Hour 24, Hour 48 Hour 72, Hour 96 and Hour 120. After centrifuging the harvested culture supernatant at 2 k rpm for 10 minutes, the supernatant was recovered. Using a kit for the HCV core antigen (Fujirebio, Inc., LUMIPULSE), 100 μL of the supernatant was subjected to measurement.

[0068]

As shown in Fig. 4, the measurement values of the core antigen in the both types cells after transfection of pTPF1-M RNA using an electroporator were highest at Hour 72 to Hour 96 after the electroporation, and the amount of the core antigen of ALS32 cells secreted was 17.5 times larger than that of Huh7/JCRB0403 cells at Hour 72 after the electroporation. Further, the amounts of HCV RNA secreted into these culture supernatants were highest at Hour 72 after the electroporation, and the amount of HCV RNA from ALS32 cells was 13.4 times larger than that from Huh7/JCRB0403 cells (Fig. 5). This indicates that the pTPF1-M gene of the present invention having the mutation introduced in the NS2 protease region can more efficiently replicate in monoclonal ALS32 cells than in their parent cells.

[0069]

5. Infection of Monoclonal Cultured Cells with HCV Particles

Whether or not the core antigen and the HCV RNA secreted into the culture supernatant in 4 form virus particles to allow reinfection *in vitro* was studied. More specifically, each of ALS32 cells and Huh7/JCRB0403 cells were transfected with full-length HCV RNA synthesized from the pTPF1-M gene, and the culture supernatant was harvested with time. The harvested culture supernatant was centrifuged at 15,000 rpm for 10 minutes and filtered (0.45μm, Millipore) to remove cell debris and the like.

[0070]

Each of the ALS32 cell-derived supernatant and the Huh7 cell-derived supernatant after filtration was reacted for 6 hours at 37°C with naive ALS32 cultured cells or naive Huh7 cultured cells, respectively, cultured in a 12-well plate (diameter, 22.1 μm). Thereafter, the cells were washed 3 times with PBS. A fresh growth medium was added to the cells, and the cells were cultured in an incubator under 5% carbon dioxide at 37°C. For evaluation of the virus infectivity titer, the core antigen accumulated in the cells was visualized by immunostaining, and the number of cells positive for the core antigen was counted, to represent the number of infective virus particles contained in the culture supernatant with the focus-forming unit (FFU/mL).

[0071]

More specifically, the cells 96 hours after the infection were fixed with methanol, and the fixed cells were incubated using 2% BSA-PBS for 2 hours at room temperature to perform blocking. Subsequently, an anti-core monoclonal antibody (1 μg/mL) as a primary antibody was added thereto, and the resulting mixture was incubated for 1 hour at room temperature. The cells were sufficiently washed, and an FITC-labeled secondary antibody was added thereto, followed by incubation of the resulting mixture for 1 hour at room temperature. HCV-infected cells were observed by counting the number of cells positive for the core antigen in the cells using an inverted fluorescence microscope.

[0072]

As shown in Fig. 6, it was confirmed that, when ALS32 cells were infected with virus particles obtained from the same type of cells, the number of infectious virus particles released into the culture supernatant was largest (1,500 FFU/mL) at Hour 96 after the transfection of HCV RNA. On the other hand, in the Huh7/JCRB0403 cells, although the number of infectious virus particles released into the culture supernatant was largest (250 FFU/mL) at Hour 48 to Hour 72 after the transfection of HCV RNA, the value was as low as one-sixth of the value in ALS32 cells. Thus, it was shown that the monoclonal ALS32 cells released a larger number of infectious virus particles than their parent cells, Huh7/JCRB0403. This indicates that the growth efficiency and the infection efficiency of the virus RNA having the mutation in the NS2 protease region can be improved by using ALS32 cells as monoclonal cells of Huh7/JCRB0403 cells.

INDUSTRIAL APPLICABILITY

[0073]

The RNA replicon of the present invention autonomously replicates by transfection into cells, and can more efficiently produce the hepatitis C virus gene, hepatitis C virus protein and infectious particles than pTPF1/4B RNA. As an *in vitro* model of hepatitis C virus infection, the replicon-replicating cell into which this replicon RNA was transfected reflects the mechanism of HCV propagation *in vivo*, and this replicon-replicating cell can therefore be used in a method for screening therapeutic drugs against HCV. Further, in addition to screening of therapeutic drugs against HCV, the screening method can be used for quality control in the production process of a therapeutic drug, so that the present method can also be used as a method for producing a pharmaceutical agent.

CLAIMS

1. A hepatitis C virus gene that encodes threonine as the 979th amino acid, leucine as the 1804th amino acid and lysine as the 1966th amino acid.

2. The gene according to claim 1, wherein the amino acid sequence encoded by said gene is the amino acid sequence shown in SEQ ID NO:2 or an amino acid sequence having a sequence identity of not less than 95% to said amino acid sequence and constituting hepatitis C virus having replication capacity and infection ability.

3. The gene according to claim 2, wherein said amino acid sequence encoded by said gene is the amino acid sequence shown in SEQ ID NO:2.

4. The gene according to claim 2, whose nucleotide sequence is the nucleotide sequence shown in SEQ ID NO:1 (wherein t is optionally u) or a nucleotide sequence having a sequence identity of not less than 90% to said sequence.

5. The gene according to claim 4, whose nucleotide sequence is the nucleotide sequence shown in SEQ ID NO:1 (wherein t is optionally u).

6. The gene according to any one of claims 1 to 5, whose genotype is the 1b type.

7. An RNA replicon comprising the gene according to any one of claims 1 to 6.

8. A cell infected with the RNA replicon according to claim 7, which cell allows replication of hepatitis C virus.

9. A hepatitis C virus particle comprising the gene according to any one of claims 1 to 6.

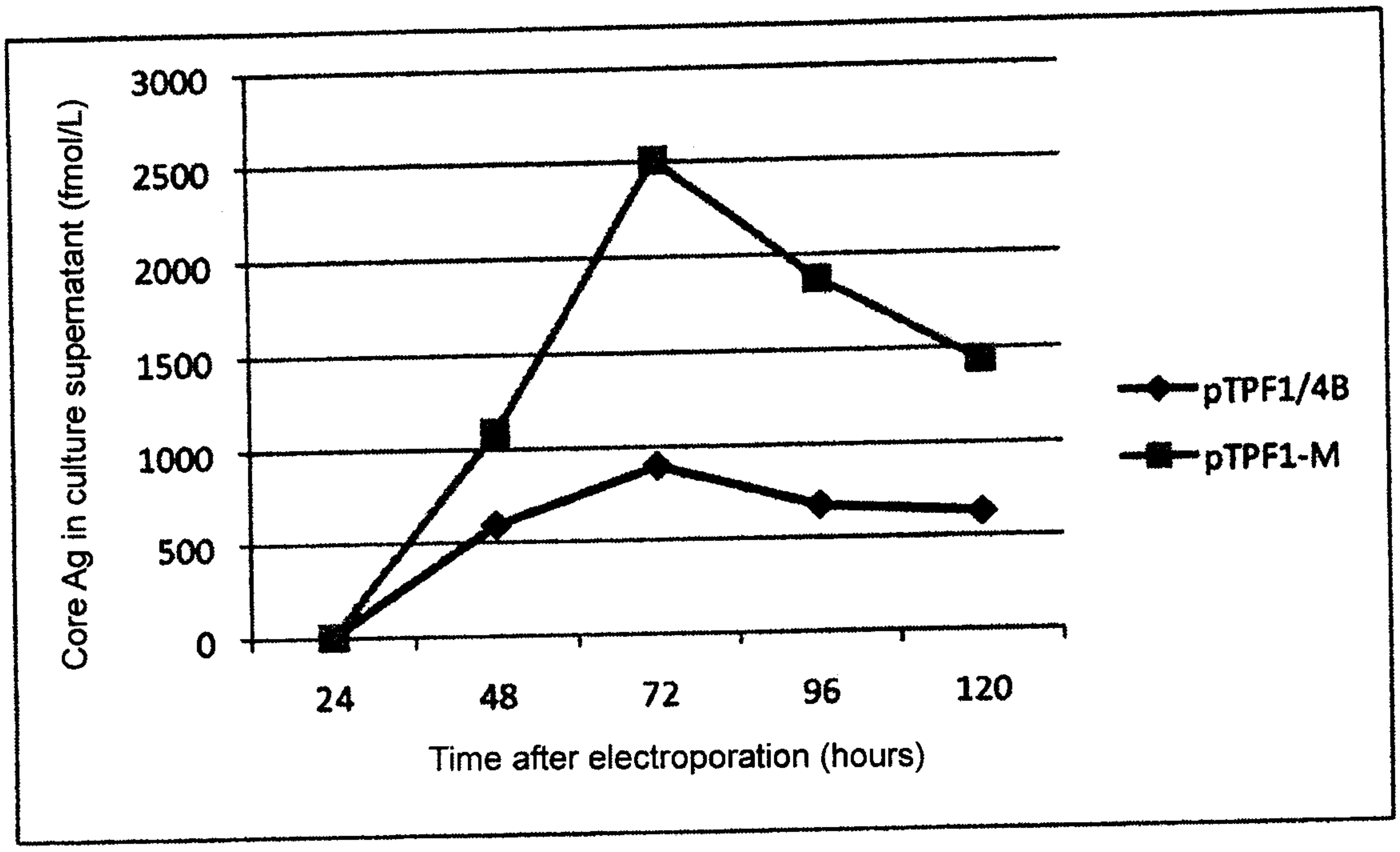

Core Ag in culture supernatant (fmol/L)
3000
2500
2000
1500
1000
500
0
24
48
72
96
120
Time after electroporation (hours)
pTPF1/4B
pTPF1-M

Fig.1

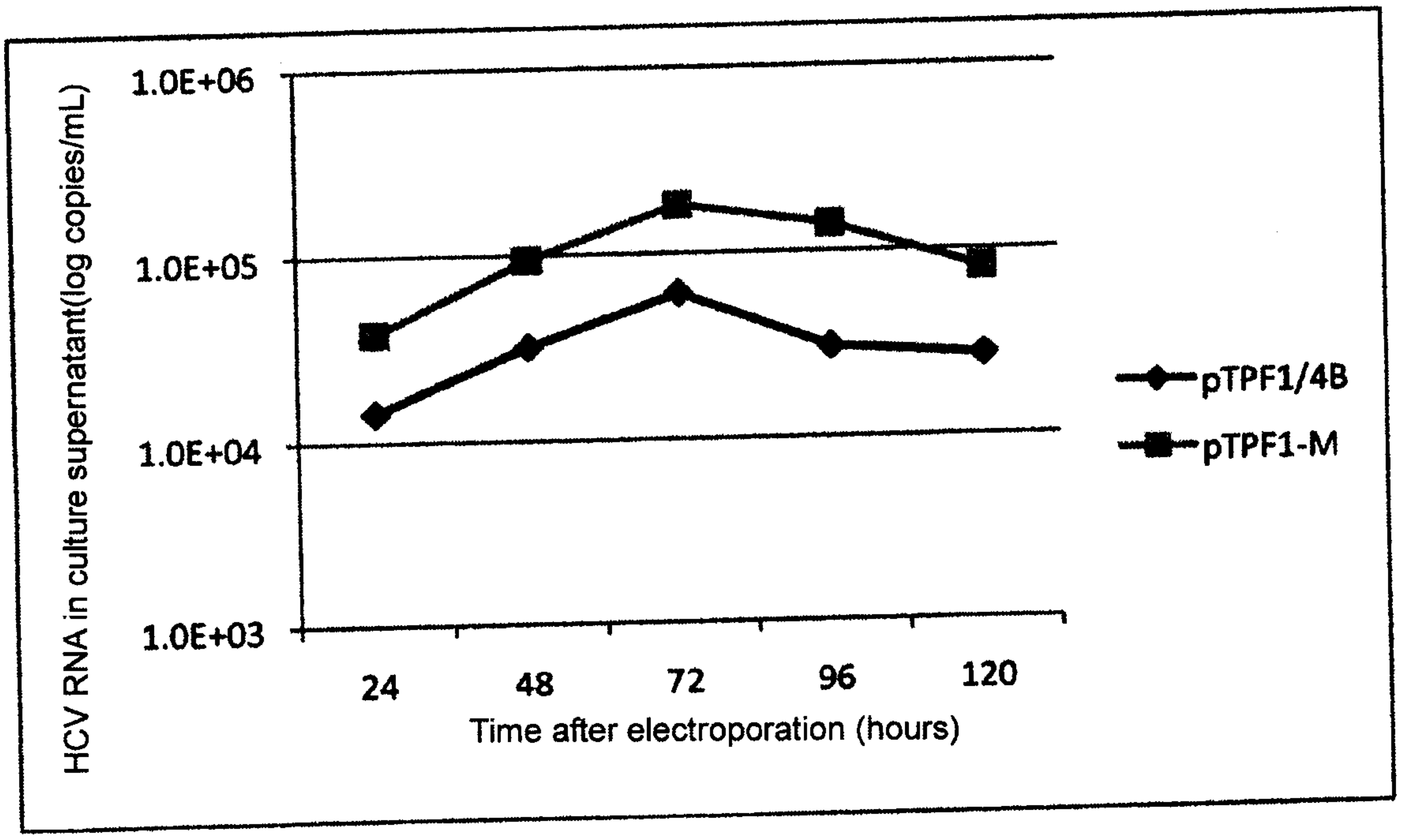

HCV RNA in culture supernatant(log copies/mL)
1.0E+06
1.0E+05
1.0E+04
1.0E+03
24
48
72
96
120
Time after electroporation (hours)
pTPF1/4B
pTPF1-M

Fig.2

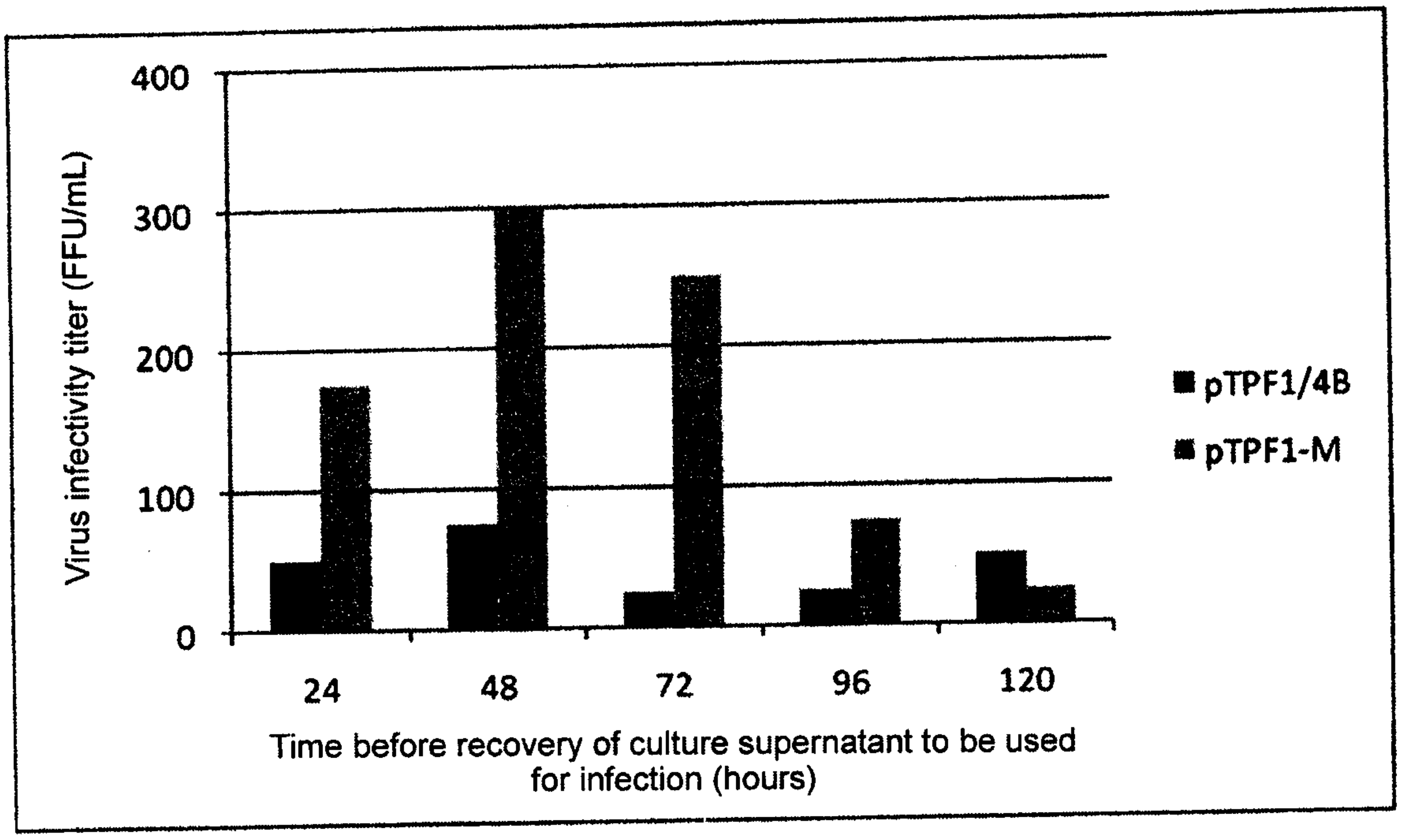

400
300
200
100
0
Virus infectivity titer (FFU/mL)
24
48
72
96
120
Time before recovery of culture supernatant to be used for infection (hours)
pTPF1/4B
pTPF1-M

Fig.3

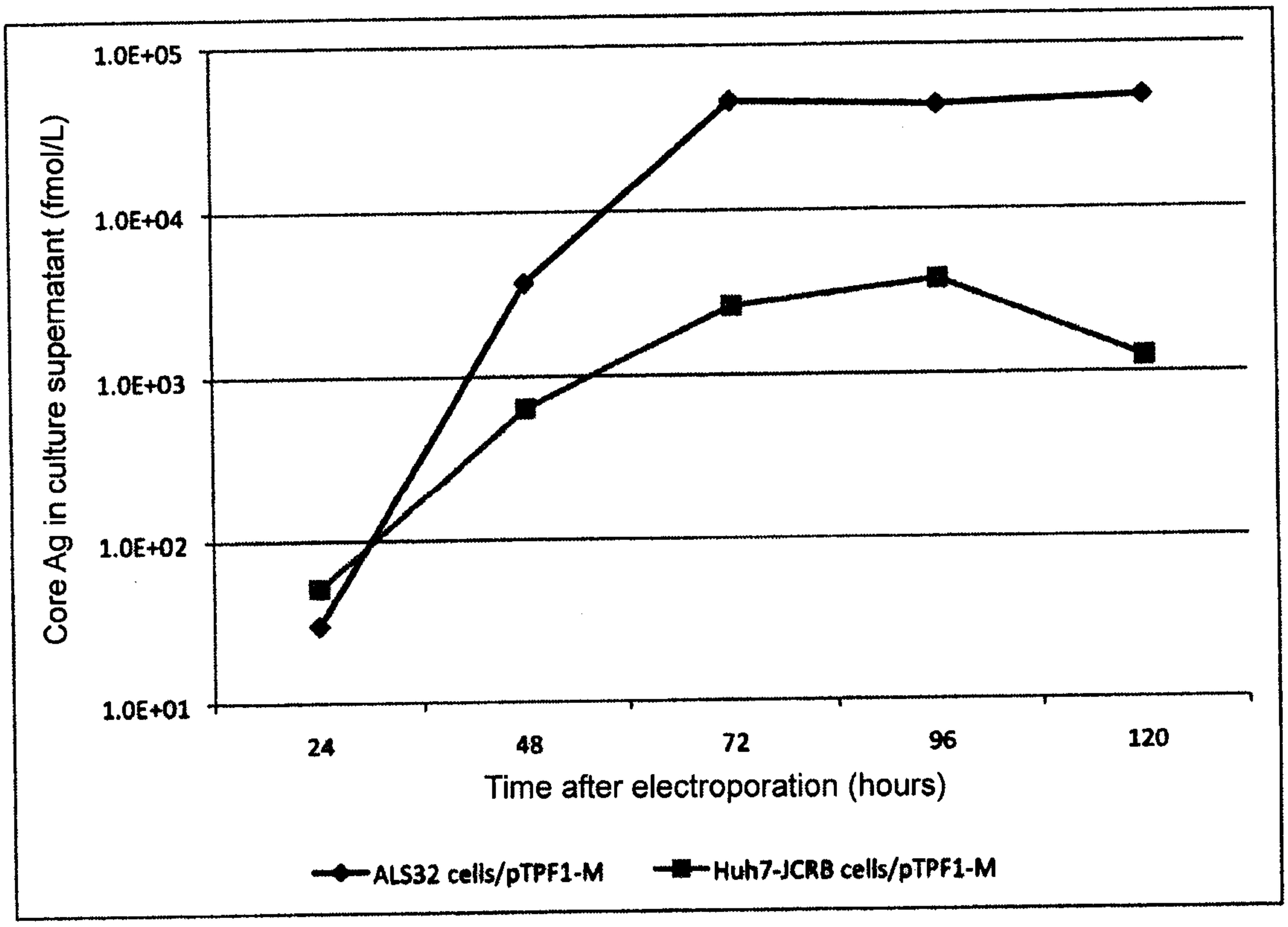

1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
Core Ag in culture supernatant (fmol/L)
24
48
72
96
120
Time after electroporation (hours)
ALS32 cells/pTPF1-M
Huh7-JCRB cells/pTPF1-M

Fig.4

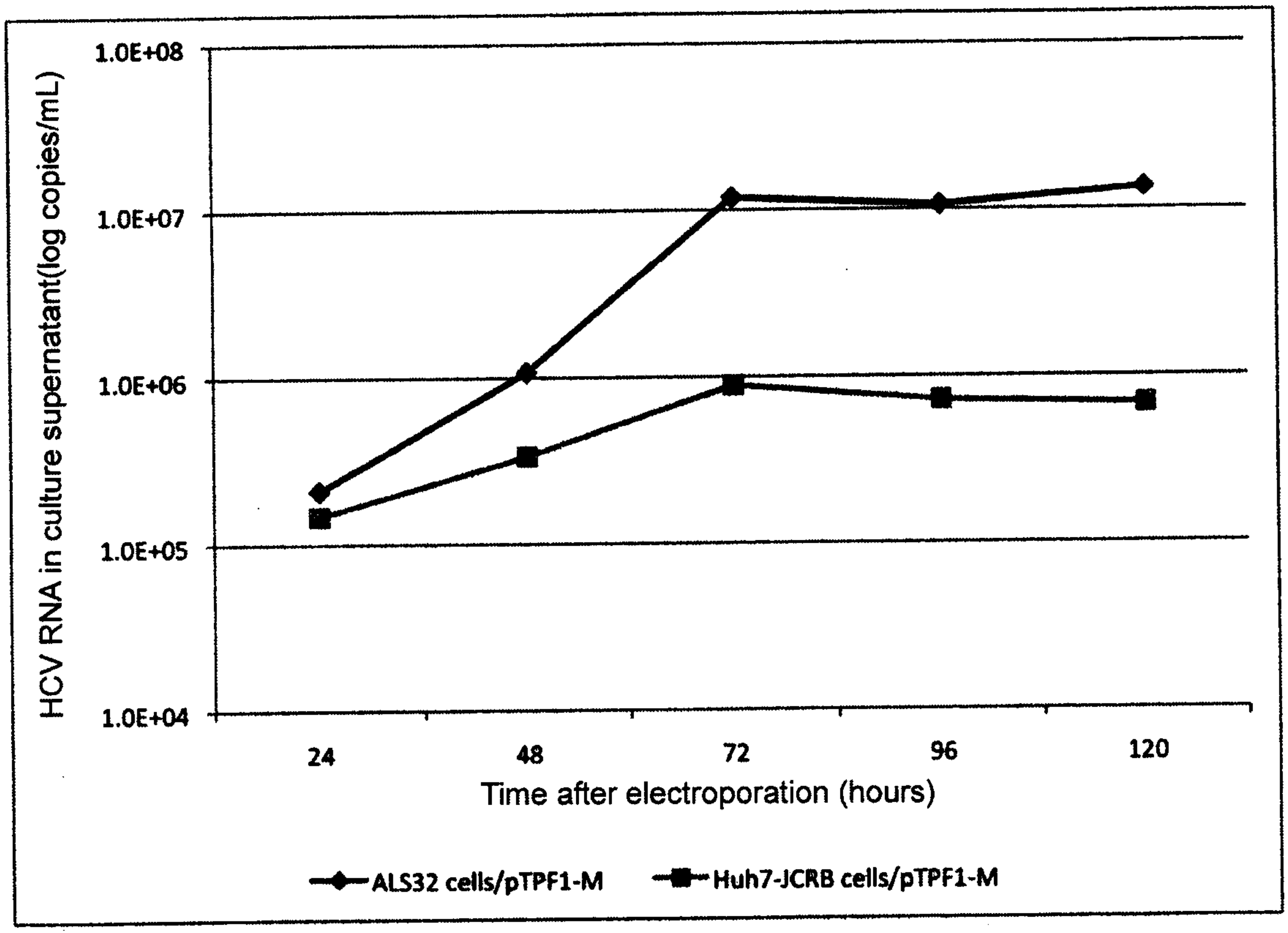

HCV RNA in culture supernatant(log copies/mL)
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
24
48
72
96
120
Time after electroporation (hours)
ALS32 cells/pTPF1-M
Huh7-JCRB cells/pTPF1-M

Fig.5

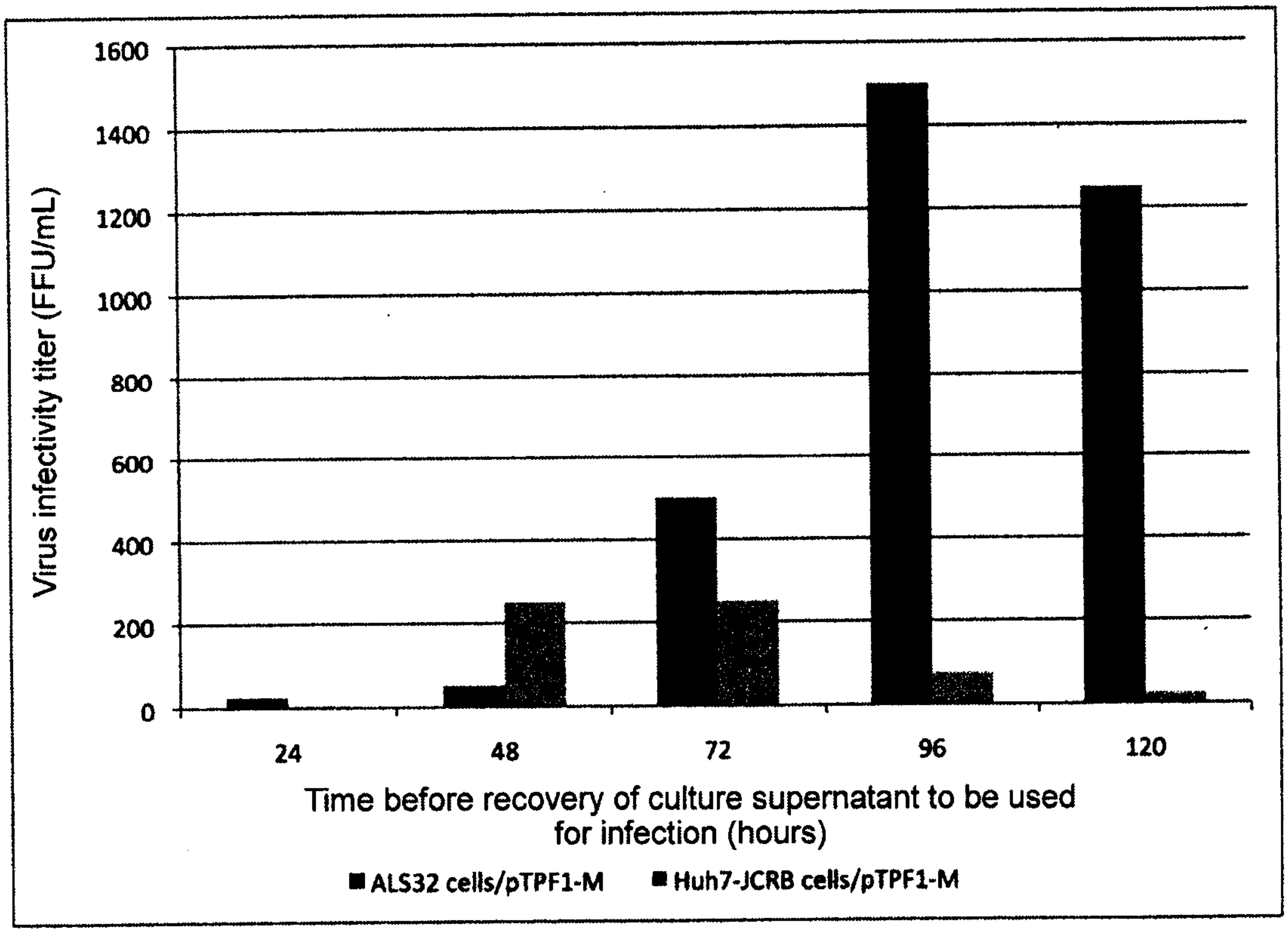
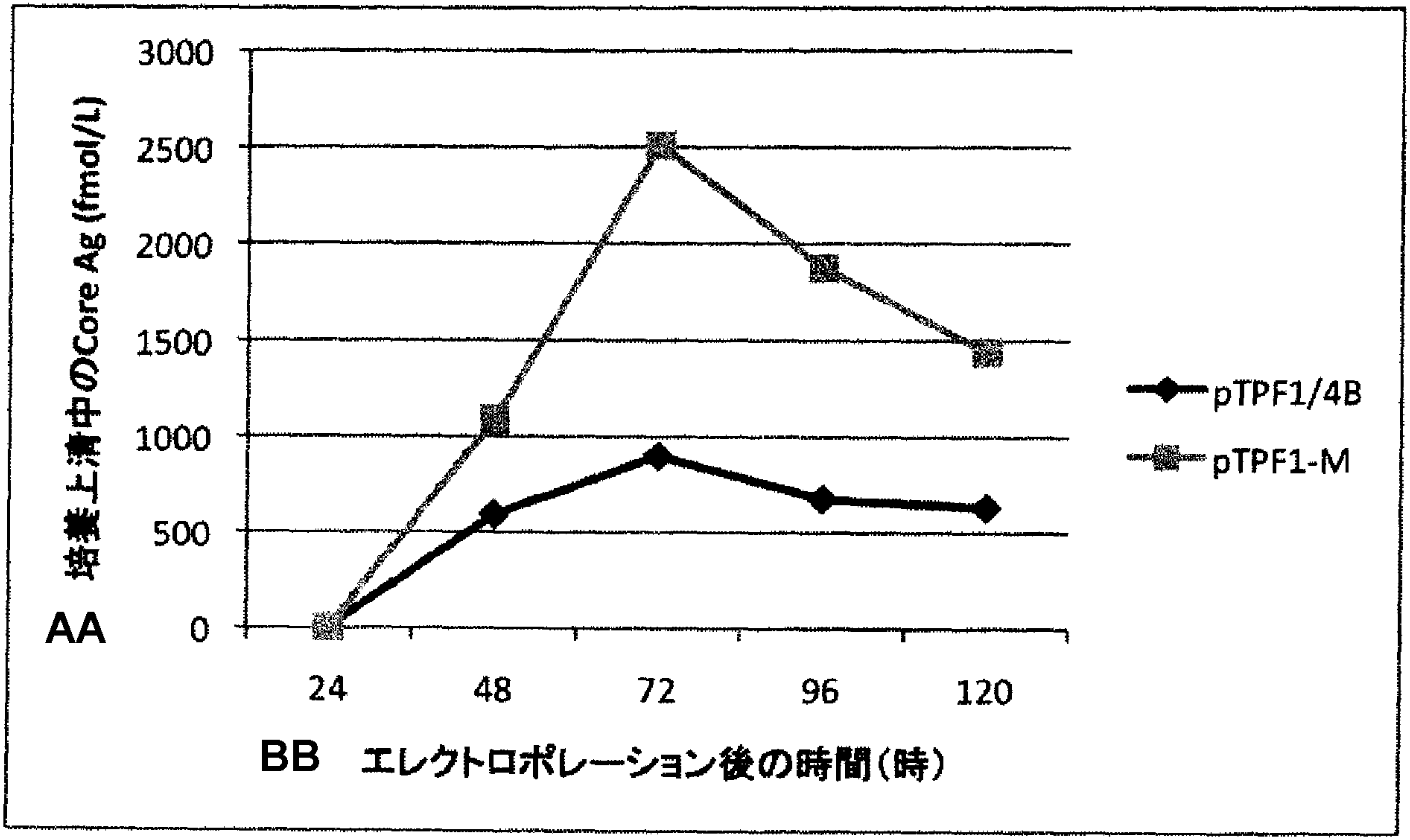

Virus infectivity titer (FFU/mL)
1600
1400
1200
1000
800
600
400
200
0
24
48
72
96
120
Time before recovery of culture supernatant to be used for infection (hours)
■ ALS32 cells/pTPF1-M
■ Huh7-JCRB cells/pTPF1-M

Fig.6

[図1]



AA Core Ag IN CULTURE SUPERNATANT (fmol/L)
BB TIME ELAPSED AFTER ELECTROPORATION (hour)