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学 位 論 文 名	感染性 cDNA クローンを用いたラット E 型肝炎ウイルスゲノムの 2 つの オープンリーディングフレーム（ORF3 および ORF4）の解析
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論文内容の要旨

1 研究目的

Hepatitis E virus (HEV) is the causative agent of acute or chronic hepatitis E, and the disease is associated with both epidemic and sporadic infections of HEV. Hepatitis E is a public health concern in many developing countries, where sanitation conditions are suboptimal. In contrast, it is now recognized as a zoonotic disease in industrial countries. Recently, a novel HEV, designated as rat HEV (ratHEV), was isolated from wild rats whose genomic sequence is markedly different from human HEV strains. RatHEV genome has four open reading frames (ORF1, ORF2, ORF3 and ORF4); the functions of ORF3 and ORF4 remain unknown. In the present study, we analyzed the functions of ORF3 and ORF4 by using infectious ratHEV cDNA clones.

2 研究方法

An infectious cDNA clone (pUC-ratELOMB-131L_wt, wt) and its derivatives, including ORF3-defective (Δ ORF3), ORF4-defective [with one (Δ ORF4-1) and three (Δ ORF4-2) premature termination codons] and replication-deficient (ORF1-GAA) mutants were constructed. Their full-length RNAs were synthesized and transfected into PLC/PRF/5 cells, and the replication activities of progeny viruses were evaluated by real-time RT-PCR, Western blotting and immunofluorescence assay (IFA). The viral particles were characterized by immunocapture RT-PCR assay. The full-length ORF4 proteins (aa 1-183) expressed in PLC/PRF/5 cells and *E. coli* were used as controls for rabbit anti-ratHEV ORF4 polyclonal antibody. In addition, seven mutants with one, two or three proline-to-leucine mutations in ORF3 (ORF3-L93, -L96, -L98, -L93+L96, -L93+L98, -L96+L98 and -L93+L96+L98) were constructed and their full-length RNAs were transfected into PLC/PRF/5 cells. The replication activities of these mutants were evaluated by the methods mentioned above.

3 研究成果

Wt, Δ ORF4-1 and Δ ORF4-2 replicated efficiently, reaching $>10^8$ copies/ml in the culture medium, while $\leq 1/1,000$ of the number of progenies were detectable in the culture supernatant of Δ ORF3-transfected cells compared with wt-, Δ ORF4-1- and Δ ORF4-2-transfected cells. The intracellular ratHEV RNA titer was nearly equal among wt-, Δ ORF3-, Δ ORF4-1- and Δ ORF4-2-transfected cells. The ORF2 protein was detectable at equal levels in wt-, Δ ORF3-, Δ ORF4-1- and Δ ORF4-2-transfected cells, while it was significantly lower in the supernatant of Δ ORF3-transfected cells than in the supernatant of wt-, Δ ORF4-1- and Δ ORF4-2-transfected cells. In immunocapture RT-PCR assay of the culture supernatants of wt-, Δ ORF4-1- and Δ ORF4-2-transfected cells, essentially no particles were captured by anti-ORF2 mAb or anti-ORF3 mAb. After treatment with deoxycholate, viral particles in the culture supernatant of wt-, Δ ORF4-1- and Δ ORF4-2-transfected cells were captured by anti-ratHEV ORF3 mAb, indicating the presence of the ORF3 protein on the surface. When treated with deoxycholate and trypsin, essentially all viral particles were captured by anti-ORF2 mAb, while none were captured by anti-ratHEV ORF3 mAb. In contrast, in the culture supernatant of Δ ORF3-transfected cells, nearly all viral particles were captured by anti-ratHEV ORF2 mAb, but none were captured by anti-ORF3 mAb. These results indicate that Δ ORF3 virus is not covered with a lipid membrane, while the wt-, Δ ORF4-1- and Δ ORF4-2 viruses are covered with membranes, and that the ORF3 protein is necessary for the release of ratHEV from infected cells.

ORF4 protein was not detectable in the lysate of cells transfected with RNA transcripts of wt or in the homogenate of livers of ratHEV-infected rats. As described above, Δ ORF4-1 and Δ ORF4-2 progeny viruses grew as efficiently as wt, despite the absence of ORF4 protein. These results indicate that ORF4 is unnecessary for the active replication of ratHEV.

Seven ratHEV ORF3 mutants with one, two or three proline-to-leucine mutations at amino acid (aa) 93, 96 or 98 of the ORF3 protein (ORF3-L93, -L96, -L98, -L93+L96, -L93+L98, -L96+L98 and -L93+L96+L98) were constructed and their full-length RNAs were transfected into PLC/PRF/5 cells. Wt and ORF3-L98 replicated efficiently and banded at a density of 1.15–1.16 g/cm³. In contrast, ORF3-L93+L96, ORF3-L96+L98 and ORF3-L93+L96+L98 replicated gradually and exhibited a decreased virus release, peaking at 1.26–1.27 g/cm³, similar to Δ ORF3. Notably, the ratHEV particles of ORF3-L93, ORF3-L96 and ORF3-L93+L98 banded at two peaks of 1.15–1.16 g/cm³ and 1.26–1.27 g/cm³. A sequence analysis revealed that revertants of ORF3-L93, ORF3-L96 and ORF3-L93+L98 were generated during cultivation of progeny viruses: leucine at aa 93 was converted to proline in the ORF3-L93 and ORF3-L93+L98 viruses; and leucine at aa 96 was converted to proline in the ORF3-L96 virus. These results indicate that proline residues at aa 93 and aa 96 play an important role in the viral egress.

4 考察

In the current study, we developed a reverse genetics system for the ratHEV strain, ratELOMB-131L, and showed that a constructed full-length cDNA clone can be used for

phenotypic studies on ratHEV via site-directed mutagenesis. We successfully constructed a total of 11 full-length cDNA clones with various mutations in ORF1, ORF3 or ORF4 that were useful in the present functional studies on ORF3 and ORF4.

With regard to human HEV, we have previously shown that the viral particles in circulating blood and culture supernatant are lipid-associated, that the ORF3 protein is present on the surface of HEV particles released from infected cells, and that the ORF3 protein is essential for virion egress. In the present study, we constructed a Δ ORF3 mutant, whose first initiation codon (ATG) was mutated to GCA, and found that Δ ORF3 virions are not enveloped, and are capturable by anti-ratHEV ORF2 mAb (not by anti-ratHEV ORF3 mAb), with or without prior treatment with deoxycholate and trypsin. Δ ORF3 exhibited a decreased virion release. Therefore, we concluded that the ORF3 protein is responsible for the envelopment of viral particles and virion egress from ratHEV-infected cells.

We previously showed that a PSAP motif in the ORF3 protein of human HEV is necessary for egress of HEV virions from infected cells. Although the PSAP motif located in the C-terminal portion of the ORF3 protein is well conserved among all *Orthohepevirus A* and *B* species, ratHEV strains, belonging to *Orthohepevirus C* species, lack the PSAP motif but harbor a proline-rich sequence (PxYPMP) present in the C-terminal region of the ORF3 protein. The PxYPMP sequence is highly conserved among ratHEV strains, which prompted us to construct seven ORF3 mutants with proline-to-leucine mutations in the PxYPMP sequence. Regarding the envelopment and virion release, ORF3-L98 showed the same features as wt and ORF3-L93+L96 and ORF3-L93+L96+L98 showed the same features as Δ ORF3. The generation of revertants [ORF3-L93 (reverted to wt), ORF3-L96 (reverted to wt) and ORF3-L93+L98 (reverted to ORF3-L98)] and the presence of lipid membranes during cultivation also suggest that the proline-rich sequence (PxYPMP) in the ratHEV ORF3 protein, especially the proline residues at aa 93 and 96, plays a pivotal role in the formation of membrane-associated ratHEV particles possessing ORF3 proteins on the surface.

Human HEV recruits tumor susceptibility gene 101 (Tsg101), a cellular factor involved in the budding of viruses, via its PSAP motif in the ORF3 protein and requires a so-called late (L)-domain function for virion release from infected cells. In future studies, the cellular factors involved in the budding of ratHEV, which binds to the PxYPMP motif in the ratHEV ORF3 protein, must be determined in order to confirm our hypothesis.

The present study showed for the first time that an additional ORF (ORF4), which is present only in *Orthohepevirus C* species, is not necessary for the *in vitro* replication of ratHEV. Although recombinant FLAG-tagged ORF4 proteins expressed in mammalian cells and recombinant His-tagged ORF4 protein expressed in *E. coli* were detectable by Western blotting and IFA using rabbit anti-ORF4 pAb, no signals were detected in the lysates of ratHEV-infected cells or in the liver homogenates of ratHEV-infected rats according to a Western blot analysis and IFA. These results indicated that ORF4 is not necessary for the replication of ratHEV. However, since our rabbit anti-ORF4 pAb used in the present study recognizes the N-terminal

portion (aa 12–29) of a putative ORF4 protein, we cannot rule out the possibility that N-terminally truncated ORF4 protein(s) may be essential for the *in vivo* replication of ratHEV. Therefore, to rule out this possibility, future studies should perform intrahepatic injection of RNA transcripts of Δ ORF4-2 cDNA clone and/or inoculation of cell culture-generated progeny viruses of Δ ORF4-2 in rats.

5 結論

The present study showed that the ORF3 protein of ratHEV is essential for virion release from infected cells and associated with lipid membranes, similar to human HEV, that the first two proline residues within the proline-rich sequence of PxYPMP (aa 93–98) in the ORF3 protein play a pivotal role for formation and release of enveloped ratHEV, and that a putative ORF (ORF4) protein is not necessary for the active replication of ratHEV at least *in vitro*.

論文審査の結果の要旨

学位論文表題：感染性 cDNA クローンを用いたラット E 型肝炎ウイルスゲノムの 2 つのオープンリーディングフレーム(ORF3 および ORF4)の解析

本論文は、ラット E 型肝炎ウイルスの 4 つのオープンリーディングフレームのうち、ヒト E 型肝炎ウイルスとは異なる特徴を持つ ORF3 およびヒト E 型肝炎ウイルスには存在しない ORF4 について、感染性 cDNA クローンおよびその変異体を作製して機能の解明を試みたものである。

この研究で申請者は、1) ORF3 を欠損したウイルスでは細胞からのウイルス産生がほとんどなくなること、2) 細胞からのウイルス産生には ORF3 の 93-98 番目のアミノ酸に存在する PxYPMP モチーフのうち 93 番目と 96 番目のプロリン残基が重要であること、3) ORF4 を欠損した 2 種類のウイルスではウイルスの産生や増殖に影響がなかったことから、ORF4 は少なくとも *in vitro* でウイルス複製には重要ではないこと、を明らかにした。

ORF4 の機能については今後、*in vivo* の実験系を用いて詳細に解析する必要があると考えられるが、本研究は多岐にわたる研究手法を駆使して行われており、得られた結果は明快であった。本研究を通して得られた結果はウイルス学的に重要な研究成果であると言え、博士の学位を授与するに相応しいものと高く評価できる。

以上のことから、本申請論文は学位論文として合格であると判定された。

なお、審査員からいくつかの修正点が指摘されたが、それらは全て適切に修正されたことが確認された。

最終試験の結果の要旨

申請者は学位論文に沿った研究内容の発表を行った。発表は日本語で行われたが、大変分かりやすく、プレゼンテーションの構成も理解しやすいように作られていた。発表時間もほぼ予定通

りであった。

申請者の発表の後に、E 型肝炎の臨床経過に関して、ウイルスの複製機構に関して、ORF3 や ORF4 の機能の解析結果の解釈等について活発な質疑応答が行われた。

主に日本語で質疑応答が行われたためか、いくつかの質問に対しては十分に質問の意図を汲みとれていなかったように感じるが、全体としては適切に質問に答えており、有意義な質疑応答が行われたと考える。

論文審査および最終試験から、申請者が研究者として十分な知識と研究遂行能力を有すると評価し、審査員全員一致で最終試験に合格と判断した。