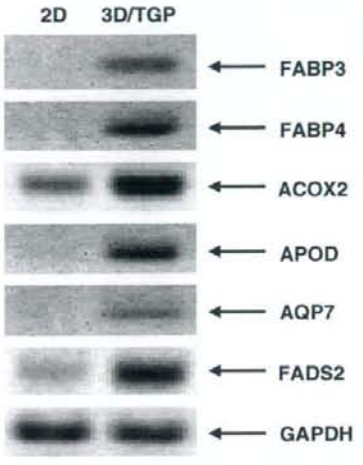

Fig. 2. RT-PCR analysis of the expression of genes identified by microarray. The PPARα regulated genes were increased in 3D/TGP-HuS-E/2 cells (3D-TGP) and their expression levels measured by RT-PCR. 2D represents RNA samples from 2D-HuS-E/2 cells. Twenty cycles of amplification were undertaken for the RT-PCR analysis. GAPDH expression served as an internal control. *Abbreviations:* FABP3, fatty acid binding proteins 3; FABP4, fatty acid binding proteins 4; ACOX2, acyl-coenzyme A oxidase 2; APOD, apolipoprotein D; AQP7, aquaporin 7; FADS2, fatty acid desaturase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

approximately 50–60% and almost completely, respectively, when compared to the replication in cells receiving mock treatment (Fig. 1C). These results demonstrate that the IFNα treatment was effective on HCV derived from RC5 and that 3D/TGP-HuS-E/2 cells may be useful for the screening of anti-HCV drugs for the treatment of natural HCV.

Increased activation of the PPARα signaling pathway in 3D cultured HuS-E/2 cells

Given that 3D/TGP-HuS-E/2 cells demonstrated enhanced proliferation of natural HCV, the gene expression profiles of these cells was compared with that of cells cultured under normal 2D conditions using microarray analysis in order to identify the factors required for the enhanced proliferation. Among the 24,268 genes compared in this analysis, 212 genes demonstrated a greater than four folds index increase in expression in 3D/TGP than standard cultured cells. Cell signaling pathway analysis of these 212 genes showed that six genes, including fatty acid binding proteins 4 and 3 (FABP4 and 3), apolipoprotein D (APOD), aquaporin 7 (AQP7), acyl-coenzyme A oxidase 2 (ACOX2), and fatty acid desaturase 2 (FADS2), were targets of PPARα signaling [9–12]. The increased expression of these genes in the 3D/TGP-HuS-E/2 cells was further confirmed by RT-PCR analysis (Fig. 2). Given that PPARα is an essential factor for normal hepatocyte function [13], these results indicate that 3D/TGP culture enhances the hepatocyte-specific characteristics of HuS-E/2 cells.

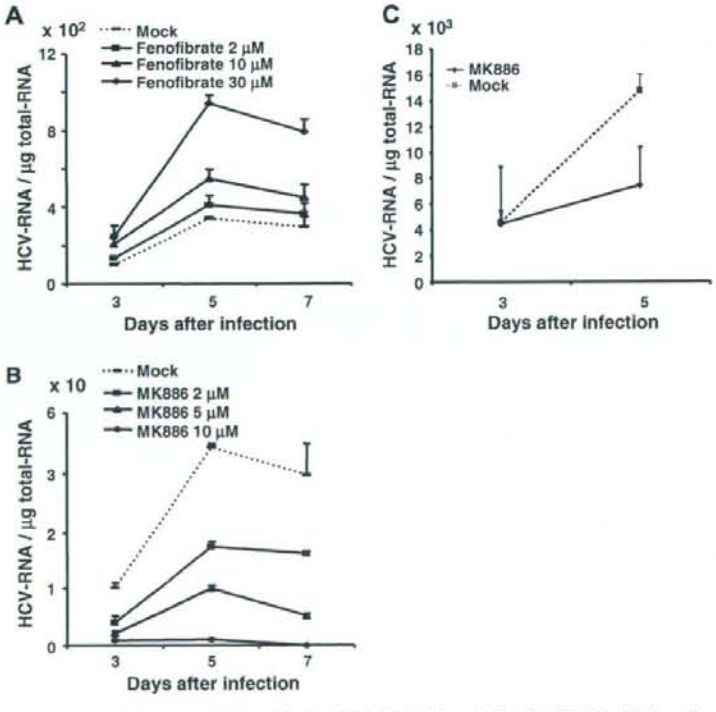

Fig. 3. The effects of PPARα agonists and antagonists on natural HCV proliferation. (A) HuS-E/2 cells were infected with HCV and fresh medium supplemented with or without (Mock) 2, 10, or 30 μM of fenofibrate overlaid on the cells. (B) Medium supplemented with or without (Mock), 2, 5, or 10 μM of MK886 was overlaid on 2D-HuS-E/2 cells infected with HCV. HCV proliferation following treatment was measured by Q-PCR. (C) Medium supplemented with or without (Mock), 10 μM of MK886 was overlaid on 3D/TGP-HuS-E/2 cells infected with HCV. HCV proliferation following treatment was measured by Q-PCR.

PPARα signaling affects HCV replication

We next examined the potential role of PPARα signaling on HCV proliferation by monitoring HCV replication in 2D-HuS-E/2 cells that had been infected with HCV-RC5 and subsequently treated with the PPARα agonist fenofibrate [14] or the PPARα antagonist MK886 [14] (Fig. 3B). As outlined in Fig. 3A, a dose-dependent increase in HCV replication was observed in fenofibrate-treated cells. In contrast, a dose-dependent decrease in HCV proliferation was observed in the presence of MK886. Similarly, treatment with MK886 reduced HCV proliferation in 3D/TGP-HuS-E/2 cells (Fig. 3C). The response of HCV proliferation in response to fenofibrate and MK886 treatment was also analyzed in LucNeo#2 cells that contained HCV replicon RNA (LNMH14) derived from the HCV-1b genome (Fig. 4A). Luciferase expression in these cells represented replication of the HCV replicon [6] and, as shown in Fig. 4A, luciferase activity in the cells treated with fenofibrate or MK886 also showed either enhancement or suppression of replicon proliferation, respectively. In addition, the increased HCV replication following fenofibrate treatment was completely abolished when treated with MK886 simultaneously. As MK886 is known to induce apoptosis when administered in high doses [15], the cell viability was examined using the XTT assay. There were no significant effects on cell viability after treatment with fenofibrate. Although MK886 resulted in a minor reduction in XTT values when high doses (10–15 μM) were administered, this reduction was not statistically significant when compared to its effect on HCV replication (Fig. 4B). This result suggests that PPARα signaling is required for HCV replication and that suppression of PPARα signaling has an anti-HCV effect.

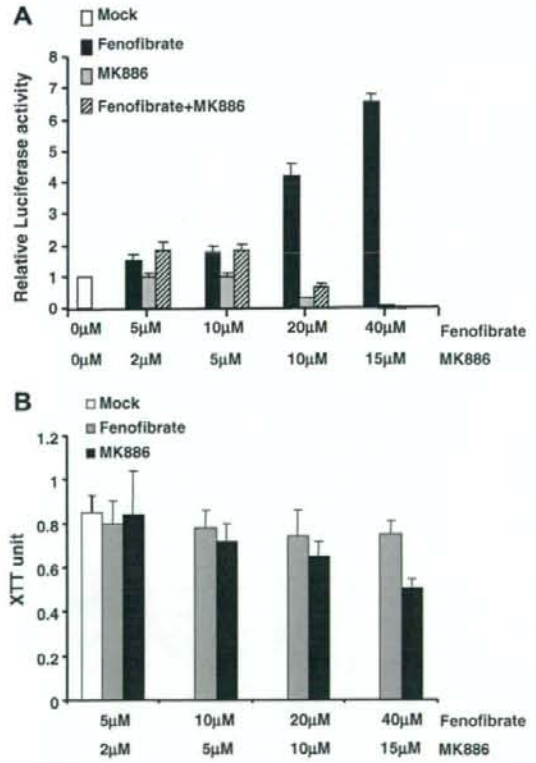

Fig. 4. The effects of PPARα agonists and antagonists on the replication of HCV subgenomic replicons. (A) LucNeo#2 cells containing a HCV subgenomic replicon termed LNMH14, were mock treated or treated with fenofibrate, MK886, or a combination of both fenofibrate and MK886 at the indicated concentrations for 2 days. Luciferase activity derived from the replicon was then measured as an indicator of HCV replication [7]. (B) Following treatment with fenofibrate and Mk886, LucNeo#2 cells were cultured for 2 days and cell viability measured using the XTT assay (Roche, Mannheim, Germany).

Discussion

In the current study, we demonstrated that immortalized hepatocyte HuS-E/2 cells cultured in 3D/TGP support the infection and replication of natural HCV derived from patient sera. Unlike recombinant HCVs, which have been required to adapt to sublines of HuH-7 cells [16], the population of the natural HCV is fairly polymorphic, demonstrating different responses to a variety of anti-viral agents [17,18]. The 3D/TGP-HuS-E/2 cells have the advantage of being a small-scale 3D cultured cells, which are cultured in 12-well plates at a density of 1×10^5/well, that allow the study of both viral and cellular events. In the current study, it demonstrated a 2 log increase in susceptibility to natural HCV infection and replication when compared to conventional 2D culture systems. Thus it offers an important advantage in the study of natural HCV infection and replication, and the response of natural HCV to anti-HCV drugs.

As the ability of HuS-E/2 cells to support infection and replication of natural HCV was greatly altered by the culture conditions, it is likely that the culture system described in our study will provide important information in regards to the cellular factors that support the HCV life cycle. The microarray study showed that the expression of some genes related to the PPARα signaling pathway were upregulated in the 3D cultured HuS-E/2 cells. Using both PPARα signaling agonists and antagonists, PPARα signaling was shown to affect infection and proliferation of natural HCV. PPARα is a ligand-activated transcription factor that is primarily expressed in tissues with high lipid metabolism including the liver, where it functions as one of three major nuclear receptors and is essential for its normal function [19]. Similar to a part of our data, a negative effect on HCV replication was previously observed in the replicon-bearing cells treated with siRNA for PPARα, with only 50% reduction of HCV-RNA [20]. In this study, even a large dose of PPARα agonist enhanced natural HCV replication in the 2D-HuS-E/2 cells for three times, despite the 2 logs enhancement of HCV proliferation in 3D/TGP culture. This implies that additional factors activated in 3D/TGP-HuS-E/2 cells may be required for the efficient HCV proliferation. Further analysis of the microarray data may provide us with further information on factors that may prove useful in the development of anti-HCV drugs.

In conclusion, the novel *in vitro* culture system combining TGP and immortalized hepatocytes described in this study demonstrated efficient support of natural HCV infection and replication. This system may be used in future virological studies to define new anti-HCV strategies. It may also prove useful for the specific design of effective individual therapy according to patient-specific strains.

Acknowledgments

This work was supported by Grants-in-Aid from the Ministry of Health, Labor and Welfare of Japan; and for scientific research from Ministry of Education, Sports, Culture, and Technology of Japan.

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Virus Research 139 (2009) 74–84

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Accumulation of LANA at nuclear matrix fraction is important for Kaposi's sarcoma-associated herpesvirus replication in latency

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ARTICLE INFO

Article history:
Received 20 July 2008
Received in revised form 12 October 2008
Accepted 14 October 2008
Available online 9 December 2008

Keywords:
KSHV
LANA
ori-P
Latency
Replication
Nuclear matrix
Replisome

ABSTRACT

The Kaposi's sarcoma-associated herpesvirus (KSHV) genome replicates once per cell cycle, and the number of viral genome is maintained in the latency. The host cell-cycle-dependent replication of the viral genome is a fundamental process to critically keep the number of the genome. Here we show that the cellular pre-replication complex (pre-RC), the viral replication origin (ori-P) in a unit of the terminal repeat of the KSHV genome, and a viral replication factor, latency-associated nuclear antigen (LANA) accumulate at the nuclear matrix fraction in the G1 phase. We found not only that LANA itself was localized mainly to the nuclear matrix fraction but also that TR region of the KSHV genome existed together in the G1 phase. The localization of LANA at the nuclear matrix could be determined by structural consequence of the full length of LANA. Furthermore, transient replication assay revealed that the LANA's nuclear matrix localization was a pre-requisite for the efficient viral genome replication in the latency. Since LANA has been shown to bind the LANA binding sites (LBS) of the ori-P, these results suggest that LANA should recruit the ori-P to the nuclear matrix, where the complete pre-RC then forms on the ori-P, during the G1 phase. Thus, the nuclear matrix accumulation of cellular and viral replication factors is likely to be a key process for the initiation of replication of KSHV in the latency.

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus associated with Kaposi's sarcoma (Boshoff and Weiss, 2001; Chang et al., 1994; Chang and Moore, 1996) primary effusion lymphomas (PEL), and multicentric Castleman's disease (Cesarman et al., 1995; Chang et al., 1994; Chang and Moore, 1996). Like all other herpesviruses, KSHV displays both latent and lytic infection. In fact, in most KSHV-infected cells, the virus is in the latent state, and the viral genome replicates according to the cell cycle, maintaining a constant number of viral genomes. Thus, the viral genome has to replicate once per cell cycle in the host cells and segregate accurately into the two daughter cells. In the latent phase, only a limited set of viral genes is expressed and among them, latency-associated nuclear antigen (LANA), one of the major latent proteins, is essential for the episomal maintenance and the replication of KSHV genome in latency (Ballestas et al., 1999; Cotter and Robertson, 1999; Hu et al., 2002).

Replication of KSHV genome is thought to be executed using host replication machinery. This includes the pre-replication complex (pre-RC), which contains ORC1–6 (ORCs), Ccd6, Cdt1, MCM1–7 (MCMs), and other factors and is set up on a replication origin prior to initiation (Bell, 2002; DePamphilis, 2003; Ohsaki et al., 2004). Previous studies have demonstrated that the viral terminal repeat region and LANA play key roles in the latent replication of KSHV (Fejer et al., 2003; Hu et al., 2002; Stedman et al., 2004; Verma et al., 2006). An interaction between LANA and ORCs has been reported to be detected by GST-ORC pull-down, immunoprecipitation followed by immunoblotting, and ChIP assays (Lim et al., 2002; Verma et al., 2006). It is, however, still unclear, how LANA is involved in the KSHV genome replication and how one viral replication origin (ori-P), which consists of LANA binding sites (LBS) and a 32-bp GC-rich segment among multiple copies of them is determined and how components of the pre-RC are specifically recruited to the ori-P region in the latency. In their model, LANA binds to the LBS in the ori-P and recruits ORCs there by direct interaction between LANA and ORCs. N-terminal deletion of LANA, which is supposed to maintain binding activity to LBS and ORCs, cannot support the viral replication (Garber et al., 2001; Lim et al., 2002; Verma et al., 2006).

As for Epstein–Barr virus (EBV), it also replicates dependent on cell cycle in latency. In this case, EBNA1 binds with its ori-P and recruits ORCs by directly binding ORCs (Chaudhuri et al., 2001; Dhar et al., 2001; Ritzi et al., 2003; Schepers et al., 2001; Sugden, 2002). And also, it was reported that nuclear matrix had a function for EBV replication in the latency and the lytic replication (Mattia et

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0168-1702/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2008.10.011

al., 1999). The detail, however, remains to be elucidated (Sugden, 2002).

Recently, the importance of nuclear matrix as the site of replication factory has been realized (Anachkova et al., 2005; Jackson and Cook, 1995), and many nuclear matrix-associated proteins have been identified (Mika and Rost, 2005; Radichev et al., 2005). It is well-known that chromatin is arranged into repeating loop domains of 50–200 kb in the interphase nucleus (Cook and Brazell, 1975; Pardoll et al., 1980; Vogelstein et al., 1980). These chromatin loops are anchored to the nuclear matrix by scaffold/matrix attachment regions (S/MARs), which bind to specific components on the nuclear matrix. It is proposed that the organization of chromatin into higher-order structure is mediated by clustering of these repeating loops. This higher-order clustering of loop domains is thought to be a fundamental feature of the functional units of chromatin in the cell nucleus. Consistent with this concept, DNA replication foci have been visualized in the nucleus of cell preparations that preserve the nuclear matrix structures (Berezney et al., 1995; Nakayasu and Berezney, 1989; Neri et al., 1992). Similarly, transcription sites in the cell nucleus are proposed to be composed of clusters of transcriptional units attached to the nuclear matrix as a transcriptosome (Cook, 1999; Jackson and Cook, 1995; Wei et al., 1999).

In this study, we demonstrated that LANA, the ori-P, and the pre-RCs were associated with the nuclear matrix and that the nuclear matrix accumulation of LANA was likely to be a key process for the initiation of replication of KSHV in the latency, suggesting that the nuclear matrix is important for the replication initiation site for the KSHV genome. Our findings suggest a model in which the ori-P is recruited to the nuclear matrix region by LANA, which accumulates there on its own, via LANA-binding sites within the TR. The LANA-bound ori-P is then ready for pre-RC placement. This model does not necessarily require LANA to interact with the pre-RC components directly, since the cellular pre-RCs itself accumulates at the nuclear matrix (Jenke et al., 2004; Radichev et al., 2005), though it does not explain necessity of GC-rich 32 bp replicator (RE) for the viral replication in the latency.

2. Materials and methods

2.1. Plasmids

pGEX-hORC1 (a gift from Dr. Hiroyoshi Ariga, Hokkaido Univ.) (Takayama et al., 2000) was digested with BamHI and SalI and inserted into the BglII/SalI site of the pEGFP-C1 vector (Clontech) to construct the pEGFP-ORC1. As for V5 tagged expression vectors, the full length of LANA ORF (vFL), and vΔCBS, in which a chromosome binding site up to 106aa was deleted, and vΔN, in which N-terminal amino acids up to 496aa was deleted, and vDBD1, which contained a DNA biding domain of LANA from 922aa to 1162aa as described elsewhere (Sakakibara et al., 2004), were fused with the V5 tag at C-terminus of each construct. EGFP tagged expression vectors, gFL, which contained the full length of LANA, and gN, which contained N-terminal 273aa of LANA, were fused with EGFP at N-terminus. gN-DBD contained the N-terminal 273aa plus C-terminal part from 922aa to 1162aa in frame and EGFP was fused with its N-terminus in this construct. gL321 contained N-terminal 107aa of LANA, and EGFP was fused to the C-terminus in this case. BSII-TR6 is a plasmid containing six TR units in the XbaI site of the pBluescirpt II vector (Stratagene).

2.2. Cells

BC3, a KSHV-positive and EBV-negative primary effusion lymphoma cell line, was grown in RPMI 1640 (Nissui, Tokyou, Japan) supplemented with 10 i.u. per milliliter penicillin G, 10 μg per milliliter streptomycin, and 20% heat-inactivated fetal bovine serum (FBS). BJAB, a KSHV-negative and EBV-negative Burkitt lymphoma cell line, was grown under the same conditions, with 10% heat-inactivated FBS.

A human embryonic kidney cell line HEK293 was grown in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) with the same supplements as used for the BJAB cells. 293LANA cells, which were obtained by introducing a retroviral vector, pHyTc-LANA, which constitutively expresses LANA, into HEK293 cells (Sakakibara et al., 2004), were cultured under the same conditions as the HEK293 cells, except that 0.2 mg/ml hygromycin B (Wako Pure Chemicals, Osaka, Japan) was added. 293hyg cells were obtained by introducing the parental pHyTc vector and cultured as the 293LANA cells. All cells were cultured in a 5% CO_2 atmosphere.

GFP-ORC1/BC3 cells were generated by introducing pEGFP-ORC1, in which an EGFP gene was followed by the full-length *orc1* gene in the pEGFP-C1 vector (Clontech) (see below), with TransFectin® (BioRad) according to the manufacturer's instructions, and cultured for 2 days. Two days after transfection, the cells were exposed to 500 μg/ml G418, cultured for 7 more days, and individual G418-resistant colonies were obtained in RPMI medium containing 0.15% methylcellulose and the same supplements as above. Isolation was repeated at least three times to obtain a completely single clone, and several independent clones were established.

2.3. Cell fractionation

Cells were fractionated as described (Belgrader et al., 1991; Payrastre et al., 1992; Radichev et al., 2005; Reyes et al., 1997). Briefly, 2×10^6 cells were harvested and suspended in 200 μl CSK buffer (100 mM NaCl, 300 mM Sucrose, 3 mM $MgCl_2$, 10 mM PIPES [pH 6.8], 0.5% Triton X-100, protease inhibitor cocktail [Sigma Cat #. P8340], 0.5 mM dithiothreitol [DTT]). After centrifugation at 10,000 × *g* for 5 min at 4 °C, the supernatant (the nucleo-cytoplasmic fraction; Sup1) was separated from the pellet, which was re-suspended in 200 μl CSK buffer and treated with 50 U/ml DNase I at 37 °C for 4 h. Ammonium sulfate was then added to this suspension to a final concentration of 0.25 M, and the sample was spun at 10,000 × *g* for 5 min at 4 °C. The supernatant from this centrifugation contained the chromatin (Sup2). The pellet was further extracted with 200 μl 2 M NaCl in CSK buffer for 5 min at 4 °C, and then subjected to centrifugation at 10,000 × *g* for 5 min. The supernatant fraction was collected and considered to contain histones and the other DNA (Sup3), and the pellet was considered to be the nuclear matrix-containing fraction. The pellet was finally solubilized in 200 μl 8 M Urea buffer (Sup4). For Western blotting, ten percent of each fraction (20 μl) was separated on an SDS-PAGE and subjected to the analysis. Each protein was probed with a respective specific antibody followed by appropriate secondary antibodies conjugated with horseradish peroxidase (HRP). The chemiluminescence image was taken as pictures with a lumino-image analyzer (LAS-3000®, Fujifilm, Co) and the band intensity was analyzed with Quantity One (BioRad). For PCR analysis of the associated DNA, each fraction was diluted in ten-fold volume of nuclear lysis buffer (Promega), and 0.2 mg/ml proteinase K and 0.1 mg/ml RNase A were added, and the mixture was incubated at 56 °C overnight. After a phenol–chloroform–isoamyl alcohol (25:24:1) extraction, the aqueous phase was precipitated with ethanol, and the precipitated DNA was suspended in TE (10 mM Tris–HCl [pH 7.6], 1 mM EDTA). The concentration was measured with a spectrophotometer (DU640, Beckman) and the final DNA concentration was adjusted to 10 ng/μl. PCR was

performed using primers: 5′-CCTGTCCCCGCGCGGGCCCG-3′ and 5′-GGCGCCCCCTTCCCTCGCTGC-3′ for TR as described (Sakakibara et al., 2004), and 5′-AGAAAGTGGATAAAAGAATAAAC-3′ and 5′-GGAGCTGTTTAGAACACTTCTGG-3′ for ORF57 region, respectively.

2.4. Indirect immunofluorescence assay (IFA)

BC3, BJAB, and BC3/GFP-ORC1 cells were harvested and washed with phosphate buffered saline (PBS). To prepare cells that preserved the nuclear matrix structure, the cells were permeabilized with ice-cold CSK buffer for 5 min and then attached to a glass slide using the Cytospin 3 system (Shandon). The dried and spread cells on the slides were treated with 50 u/ml DNaseI in CSK buffer at 37 °C for 15 min in a humidified box, and then with 0.25 M $(NH_4)_2SO_4$ in the buffer, to stop the reaction. Next, the cells were treated with 2 M NaCl in the CSK buffer for 5 min at RT and then fixed with 4% paraformaldehyde in PBS (4% PFA-PBS). After being washed three times for 5 min each with PBS containing 0.1% Tween 20 (Sigma, cat#: P9416) (PBS-T) and then dried, the cells were incubated with the first antibodies overnight at room temperature (RT). After another round of PBS-T washes, the cells were incubated with the secondary antibodies. The secondary antibodies used in this experiment were goat anti-mouse IgG Fab fragment antibodies conjugated either with Alexa® 488 or 546 and goat anti-rabbit IgG Fab fragment antibodies conjugated either with Alexa® 488 or 546 dependent on the first antibodies. In cases where the DNA was preserved, it was counter-stained with 4′,6′-diamino-2-phenylindole (DAPI) (Molecular Probes).

To visualize LANA and its mutants in transfected cells, 2 days after transfection of the expression vectors, the cells were fixed in 4% PFA-PBS and permealized with PBS containing 0.1% Triton X-100 for 30 min. EGFP tagged proteins were detected with its fluorescence and V5 tagged ones were probed with an anti-V5 antibody (Nakalaitesq) followed by secondary antibodies conjugated with Alexa® 488 mentioned above. DNA was counterstained with DAPI.

2.5. Cell synchronization and fluorescence-activated cell sorting (FACS) analysis

Mimosine (200 μM) (Calbiochem) was used to synchronize the BC3 and BJAB cells. Fourteen hours later, the cells were released into ordinary medium. They were harvested 0, 3, and 9 h later, washed with PBS, and then fixed with 70% ethanol at 4 °C overnight. The cells were then treated with 50 mM sodium citrate containing 100 μg/ml RNaseA, incubated at 37 °C for 2 h, suspended in 500 μl FACS flow solution containing 125 μg/ml propidium iodide (Nakalaitesq), and analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

2.6. Transfection

In a transient replication assay, HEK293 cells (2×10^6 per 3 cm dish) were transfected with 1 μg either LANA or its deletion mutant expression vectors with 1 μg BSII-TR6 using Superfect® (Qiagen). Forty-eight hours post-transfection, the cells were harvested and the Hirt DNA was prepared. The DNA was digested either with XhoI or XhoI plus DpnI and subjected to Southern blotting analysis with a TR fragment as a probe.

2.7. Antibodies

Goat polyclonal antibodies against ORC1 (Abcam, ab10876), ORC4 (Abcam, ab9641), and Cdt1 (Abcam, ab14676) were purchased from Abcam (Cambridge, UK). Rabbit polyclonal antibodies against Histone H2B (−371) and H2A (−146) were purchased from Upstate (New York, USA). Mouse monoclonal antibodies against Mcm7 (4B4), ORC2 (3B7), and GFP (RQ2) were purchased from MBL (Nagoya, Japan), an anti-Mcm5 antibody (CRCT5.1) was from Cosmo Bio (Tokyo, Japan), an anti-NuMA antibody (Ab-2) was from Oncogene (SanDiego, USA), an anti-CDC6 antibody (C0224) was from Sigma (Saint Luis, USA), an anti-V5 antibody (V5005) was from nakarai tesque (Kyoto, Japan) and a rat monoclonal antibody against LANA (LN53) was from Advanced Biotechnologies Incorporated (ABi,) (Columbia, USA).

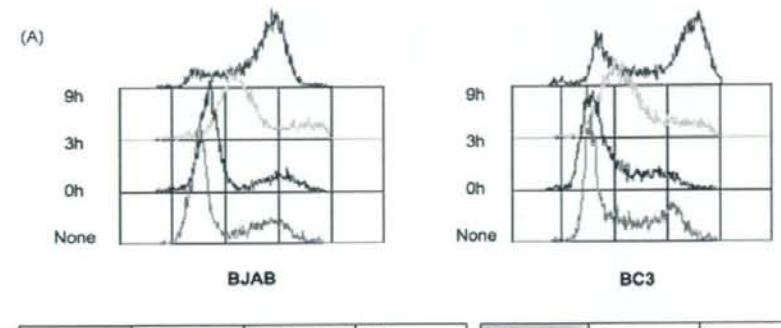
3. Results

3.1. LANA and pre-RC components are localized in the nuclear matrix fraction in G1 phase

ORC1, a component of pre-RC, must be present to complete the pre-RC formation. ORC1 joins the ORC2-6 complex on the replication origin only during the G1 phase, and thereafter it is released from chromatin during the G1-to-S transition; it is then ubiquitinated and degraded (Li and DePamphilis, 2002; Ohta et al., 2003). ORC1 is reported to enter the sub-nuclear insoluble fraction called nuclear matrix and to join the ORC2-6 complex bound to replication origins in the late G1 phase, to initiate origin replication firing (Ohta et al., 2003). Thus, a proper time and place are required for ORC1 to execute its key role in replication origin activity.

Since previous study reported that LANA interacts with all kinds of ORCs (Lim et al., 2002; Verma et al., 2006), we also tried many times to show the physical interaction of them with immunoprecipitation followed by immunoblot, but failed. Then, we constructed a plasmid expressing GFP-tagged ORC1 (GFP-ORC1) to more easily detect ORC1 biochemically and histologically. We transfected BC3 cells with this plasmid, and established several stable cell lines expressing GFP-ORC1 (GFP-ORC1/BC3) and observed that GFP-ORC1 was colocalized with LANA (Fig. 2C, and see below). We thought that ORC 1 and the other pre-RC components present at the active replication origin might be hard to be solubilized. To test the nuclear localization of the pre-RC, we performed a cell fractionation experiment and detected the GFP-ORC1 in the nuclear matrix fraction (Fig. 2B).

From the results, to test whether LANA was localized to the nuclear matrix fraction in the G1 phase, we performed cell fractionation using cells synchronized with Mimosine (Fig. 1A). BC3 and BJAB cells were incubated with 200 μM Mimosine, which arrests cells at the G1/S boundary. After 14 h, then they were released into Mimosine-free medium, harvested at the indicated times (G1 [0H], S [3H], and G2/M [9H]), and fractionated into nucleo-cytoplasmic (Sup1), chromatin (Sup2), histones containing the other DNA (Sup3), and nuclear matrix fractions (Sup4). Immediately after the release, about 58% of BC3 cells and 64% of BJAB cells were in the G1 phase (G1 [0H]); 3 h later, 56% of BC3 cells and 61% of BJAB cells had entered the S phase (S [3H]); 9 h after release, 58% of the BC3 and 56% of BJAB cells had entered the G2/M phase (G2/M [9H]), respectively (Fig. 1A). Cell fractionation followed by Western blotting analysis was carried out under these conditions (Fig. 1B), and we detected several replication factors in the fractions. In both of BC3 and BJAB cells, ORC1, Cdc6, and Cdt1 were in the nuclear matrix fraction in the G1 phase, and in the S phase at obviously lower levels (lanes 1–4 of BJAB and BC3 panels in Fig. 1B). LANA seemed to be enriched at the nuclear matrix throughout the cell cycle. Some extra bands of ORC1 and Cdt1 in the nucleo-cytoplasmic fraction may have been modified or degraded forms of these proteins (Fig. 1B). The change in protein level at the nuclear matrix fraction in each phase was calculated by normal-



BJAB	G1	S	G2M
None	53	20	27
0h	64	15	20
3h	14	61	25
9h	17	27	56

(%)

BC3	G1	S	G2M
None	48	24	28
0h	58	25	17
3h	23	56	21
9h	22	20	58

(%)

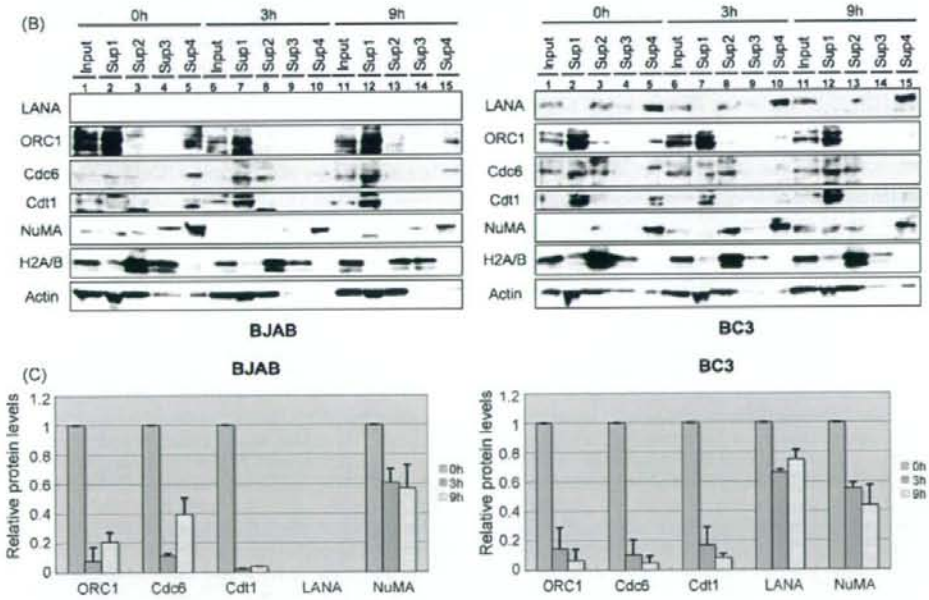


Fig. 1. LANA and pre-RC components are present in the nuclear matrix fraction in a cell-cycle-dependent manner. (A) BC3 and BJAB cells were arrested by incubation in the 200 μM mimosine containing culture medium for 14 h and then released into medium without mimosine. Cells were harvested 0, 3, and 9 h after release and analyzed by FACS. The population of each phase was analyzed by the FACSCalibur system (Beckton–Dickinson) and is shown below. (B) Western blotting analysis of the cell fractions. Samples containing 1×10^6 cells of each phase shown in (A) were fractionated as described in Section 2. Ten percent of each fraction (20 μl) was separated by SDS-PAGE and analyzed by Western blotting with the specific antibodies indicated on the left. In Input, 2.5% of each fraction was combined and analyzed in the same way. This experiment was repeated several times and a typical result is represented. (C) Accumulation pattern of ORC1, CDC6, Cdt1, LANA and NuMA in the Sup 4 fraction (nuclear matrix) was graphed. The band intensity of ORC1, CDC6, Cdt1, LANA and NuMA in the Sup4 lane and that of actin in the input lane at 0, 3, and 9 h after release of mimosine, respectively, was analyzed with a Quantity One® software (BioRad). Each Sup 4 band intensity to that of actin in the input lane at each time point after release of mimosine, respectively, was set at 1. Average from three experiments was calculated and shown with the standard deviation.

izing the values to the signal of actin in input lane at each time point after release (Fig. 1C). In the S phase, the level of LANA in the nuclear matrix fraction decreased marginally compared to that in the G1 phase. The level of NuMA also marginally decreased in the S and the G2/M phase. Because NuMA plays different roles, one of which is to regulate centrosme function, during the cell cycle, therefore the modification and/or the localization of NuMA changes. In contrast, the levels of pre-RC components drastically decreased in the S phase (lane 10 of BJAB and BC3 panels in Fig. 1B and C).

Nuclear matrix mitotic apparatus (Numazaki et al., 1998), which is one of the nuclear matrix components and has an important role in the formation of the spindle pole (Gehmlich et al., 2004) in the G2/M phase, was mainly present in the nuclear matrix fraction

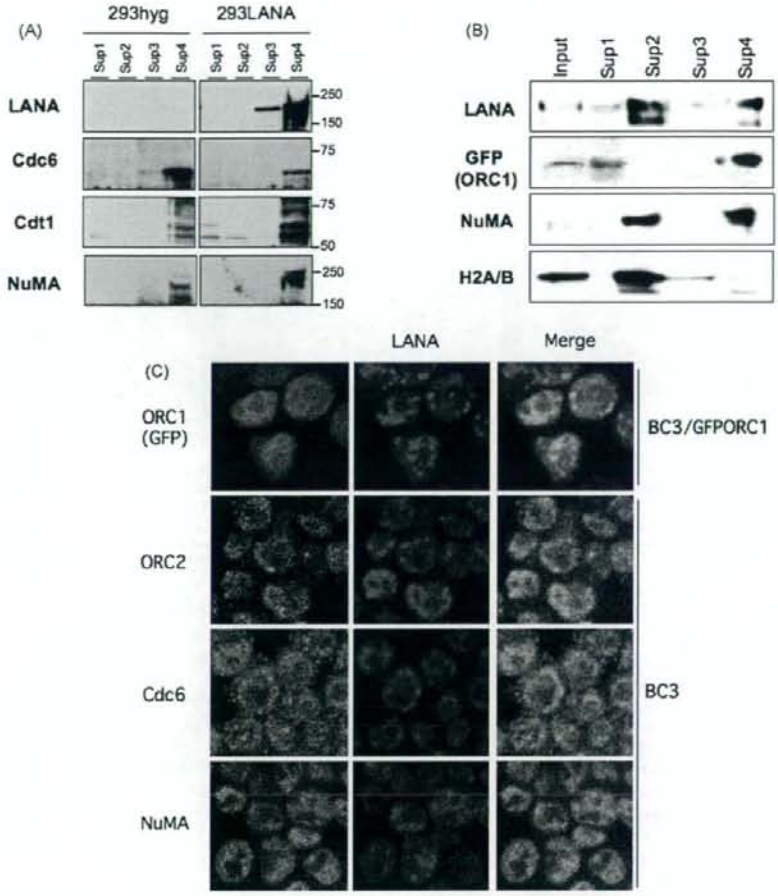


Fig. 2. LANA self-associates with the nuclear matrix fraction and colocalizes with host pre-RC components. (A) One million 293hyg or 293LANA cells were harvested and fractionated. Ten percent of each fraction was separated on 10% SDS-PAGE and subjected to Western blotting analysis. (B) One million GFP-ORC1/BC3 cells were harvested and fractionated and subjected to Western blotting analysis as in (A). (C) Colocalization analysis of LANA and pre-RC components by immunofluorescent analysis. BC3 and GFP-ORC1/BC3 cells were permeabilized with ice-cold CSK buffer for 5 min, then placed on a glass slide and prepared to preserve the nuclear matrix. Components of the Pre-RC such as Cdc6, Cdt1, and a nuclear matrix protein, NuMA were detected with specific antibodies against them. ORC1 was detected with an anti-GFP antibody in GFP-ORC1/BC3 cells. LANA is shown in red and others are shown in green. Pictures were taken with a laser confocal microscopy (Radiance® 2000, BioRad). The original magnification was 10 × 40. Note that most of the cells in the ordinary culture are in the G1 phase and most of proteins outside of nuclear matrix were depleted in this treatment.

(lanes 5, 10, and 15 of BJAB and BC3 panels in Fig. 1B), which confirmed that cell fractionation was appropriately performed, though core histones, H2A and H2B, were mainly detected in the chromatin fraction in our condition probably due to somewhat severe DNase I treatment (Fig. 1B).

3.2. *LANA localizes to the nuclear matrix fraction in the absence of other viral factors*

The above data showed that LANA was preferentially located in the nuclear matrix fraction throughout the cell cycle in KSHV-infected cells. The next question was whether LANA was recruited there by the other component, such as the viral genome. To answer this question, we performed cell fractionation in HEK293 cells stably expressing LANA (Fig. 2A). The results showed that LANA was localized mainly to the nuclear matrix fraction, and the nuclear matrix localization of Cdt1, Cdc6 and NuMA was consistent with the experiment using BC3 and BJAB cells. These results indicated that LANA localized to the nuclear matrix fraction by itself, in the absence of other viral components. In the GFP-ORC1/BC3 cells, the similar results were obtained in the cell fractionation experiment (Fig. 2B). From this finding we predicted that the TR region would be recruited to the nuclear matrix by LANA, thereby allowing the KSHV genome to initiate replication at the nuclear matrix, with the complete pre-RC. If this is the case, the TR including the ori-P should also be present in the nuclear matrix region (see below).

3.3. *LANA colocalizes with pre-RC components at the nuclear matrix*

To demonstrate LANA's association with the pre-RC at the nuclear matrix, we carried out an immunofluorescence assay of nuclear matrix preparations using a confocal fluorescence

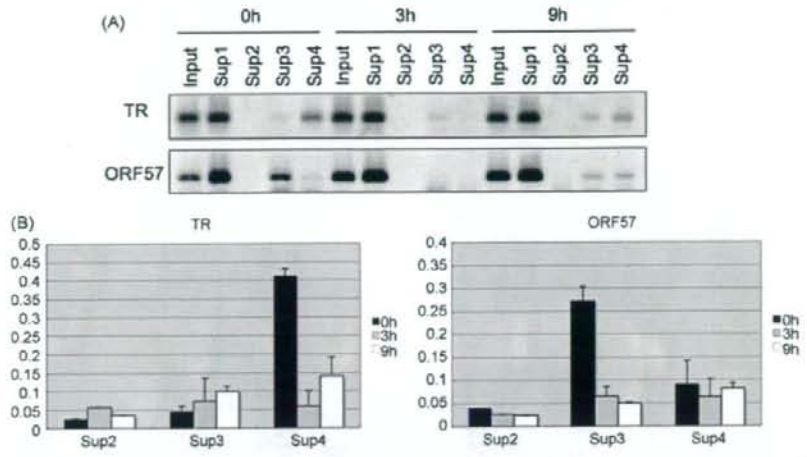


Fig. 3. The TR region (ori-P) is present in the nuclear matrix in a cell-cycle-dependent manner. DNA from fractions of the BC3 cells prepared in Fig. 1C was extracted as described in Section 2. The amount of DNA was measured by spectrophotometer (DU640, Beckman) and was adjusted to 10 ng/μl. Input DNA was prepared as a mixture of equal volume of the sup 1, 2, 3, and 4 fractions adjusted to 10 ng/μl. PCR was performed using 30 cycles for the TR and 40 cycles for ORF57, using 10 ng of each DNA. (A) The amplified samples were separated on the 2% agarose gel and visualized with ethidium bromide staining. (B) The band intensity was measured with a FX laser scanner (BioRad) and a QuantityOne® (BioRad) software. The data was calculated as the band intensity to that of the input, which was a mixed sample of all four fractions.

microscopy (BioRad Radiance® 2000). In this case, the cells were in the ordinary culture condition and most of the cells were in the G1 phase (data not shown). Cells were treated to preserve the nuclear matrix structure (see Section 2), and then stained for LANA, ORC2, GFP-ORC1, and NuMA using specific antibodies against them. No DNA was stained with DAPI, showing that most of the DNA integrity was destroyed and the nuclear matrix preparation was done well (data not shown). As shown in Fig. 2C, LANA was localized to the perinuclear region in a dotted pattern. The co-localization of LANA with components of the pre-RC, such as ORC1, ORC2, and Cdc6, was observed mainly at the perinuclear region. NuMA, a nuclear matrix protein, was also observed in the perinuclear region and co-localized with LANA, although it was stained more diffusely than LANA. These results suggested that LANA was co-localized with pre-RC components at the nuclear matrix.

3.4. The TR region is predominantly located in the nuclear matrix fraction in a cell-cycle-dependent manner

As described above, if LANA accumulated in the nuclear matrix region without being recruited by other viral components and co-localized with the pre-RC, the viral replication origin should also go to the same region to replicate, since LANA binds to the LBS within the TR and supports the replication. Fraction-associated DNA was extracted from the cells synchronized with mimosine as in Fig. 1A. All the cellular materials were exposed to DNaseI except for the nucleo-cytoplasmic fraction (Sup1), which was the DNase I pre-treatment fraction. The DNA resistant to DNase I treatment in each fraction was then treated with proteinase K followed by phenol–chloroform–isoamyl alcohol extraction and ethanol precipitation. The DNA concentration was adjusted to 10 ng/μl, and 10 ng of the DNA of each fraction was subjected to PCR. The results showed the TR region containing ori-P was present in the nuclear matrix fraction (Sup4), especially during the G1 phase (Fig. 3A and B). The ORF57 region was analyzed as a control, but it was barely detectable in the nuclear matrix fraction (Sup4) at any phase (Fig. 3A and B) and was consistently detected in the histone-DNA fraction (Sup3) in the G1 phase, although it is unclear why the sequence around ORF57 was detected in the fraction in the G1 phase. Even if the condition used in this experiment was not informative for quantitative analysis, it might be still suggestive for that TR region was detected in Sup4 at the G1 phase. Thus, remarkable difference in accumulation profile of TR and ORF57 region in the G1 phase suggests that the ori-P uniquely resides at the nuclear matrix but not all of the viral genome. Thus, the viral genome around the TR region predominantly existed in the nuclear matrix, especially during the G1 phase.

3.5. The nuclear matrix localization of LANA might be determined by the whole structural consequence

We thus elucidated that LANA was predominantly present at the nuclear matrix and TR containing ori-P was also recruited there. Then, we tested whether the localization of LANA and ori-P coincidentally happened or not. Firstly, we made several deletion mutants of LANA to determine which region of the LANA open reading frame was a minimum requirement to accumulate in the nuclear matrix fraction (Fig. 4A). As shown in Fig. 4B, only the full length of LANA was accumulated in the nuclear matrix fraction (Sup4). Either the N-terminal or the C-terminal part was not enough to nuclear matrix localization and neither was the N-terminal plus C-terminal part. In IFA (Fig. 4C), N-terminally deleted LANA (vΔCBS and vΔN) was localized in the cytoplasm. Further deletion up to 921aa (vDBD) restored its nuclear localization, but still not in the nuclear matrix fraction (Fig. 4B). We observed similar localization gFL, gN and gN-DBD in the other cell lines such as Vero and HuH7 cells. Thus, strong nuclear localization signal should be in the N-terminus (1-106aa) and in the C-terminus (922-1162aa), but they were not enough for localization at nuclear matrix fraction. Therefore, our data suggest that not a typical signal but the structural consequence of the whole protein could be a determinant for LANA's localization at nuclear matrix.

3.6. Viral replication in the latency is dependent on nuclear matrix localization of LANA

Next, we investigated the relationship between the nuclear matrix localization and the replication in a transient replication assay. A TR-containing plasmid was transfected to HEK293 cells with various LANA deletion mutants shown in Fig. 4A and the Hirt DNA was collected 48 h post-transfection and subjected to Southern blotting analysis with or without Dpn I (Fig. 4D). The results showed that decrease in the nuclear matrix localization of LANA led to drastic reduction of the replication of ori-P containing plasmid, even though some constructs (vDBD and gN-DBD) showed nuclear localization and retained LBS-binding activity (Garber et al., 2001; Komatsu et al., 2004). Thus, nuclear matrix localization of LANA could be a pre-requisite condition for KSHV replication in the latency.

4. Discussion

The KSHV genome exists as an episome in latently infected cells and maintains this condition while replicating in concert with the cell cycle. KSHV replication in latency seems to be mainly dependent on the cellular replication machinery and two viral factors, LANA and ori-P. In case of de novo infection, it probably takes a time to establish latency and start host cell cycle dependent viral replication, since the viral particles does not contain LANA (Bechtel

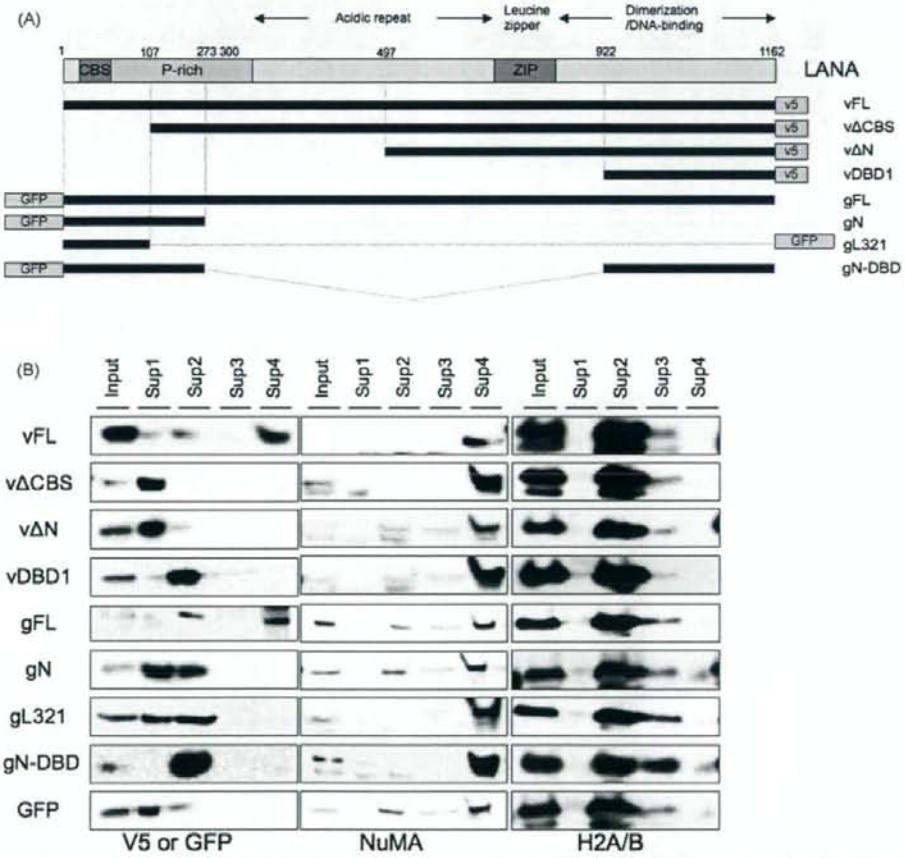


Fig. 4. Nuclear localization of LANA is required for ori-P mediated viral replication. (A) Schematic presentation of LANA expression constructs. Two full-length LANA expression vector was constructed. One was tagged at the C-terminus with a V5 epitope (vFL) and another was at the N-terminus with a GFP ORF (gFL). N-terminally deleted mutants tagged at C-terminus with a V5 epitope were vΔCBS, vΔN, and vDBD, and in each construct, N-terminal 106aa, 464aa, and 921aa were deleted, respectively. gN contained GFP tagged N-terminal 273aa of LANA and gL321 did N-terminal 107aa followed by a GFP ORF, and gN-DBD did N-terminal 273aa plus C-terminal 922 to 1162aa. (B) Cell fractionation experiment of the LANA mutants. Each expression vectors including a GFP expression vector were transfected into HEK293 cells and 2 days post-transfection, the cells were harvested and subjected to cell fractionation. The expression of LANA mutants was tested with an anti-V5 antibody or an anti-GFP antibody. Cellular fraction was assured by checking localization of NuMA and Histone H2A/B (H2A/B). In input lane, 2.5% (5 μl) of each fraction was combined and analyzed. (C) Cellular localization of LANA mutants. Transfected cells as in (B) were also subjected to IFA. LANA and its mutants were detected either by GFP or an anti-V5 antibody shown in green. Counterstained DNA was shown in red. (D) Ori-P mediated transient replication assay with LANA mutants. The Six-mer of TR containing plasmid (BSII-TR6) was transfected into HEK 293 cells with LANA expression constructs shown in (A). Forty-eight hours post-transfection, the cells were harvested and Hirt DNA was prepared. The DNA was digested with BglII completely and nine-tenth of the aliquot was further digested with DpnI. (upper panel) DpnI resistant DNA was detected with a TR fragment as a probe along with the sample without DpnI digestion (transfection control). A typical autoradiography is shown. (lower panel) Replication efficiency was calculated by setting the band intensity of DpnI (+)/(−) in case of vFL expression at 1 from three Southern blotting analyses as shown in the upper panel. The experiment was performed three times and the data are shown as the average with the standard deviation.

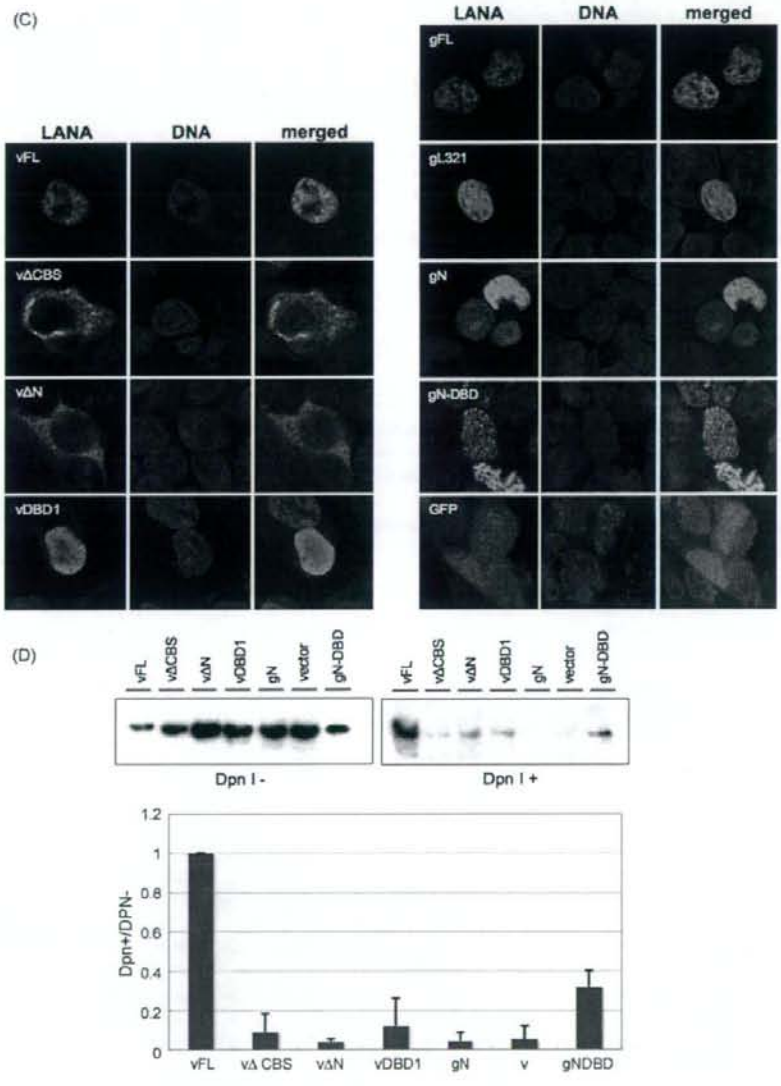


Fig. 4. (*Continued*).

et al., 2003, 2005; Grundhoff and Ganem, 2004; Lagunoff et al., 2002).

To initiate the genome replication, a complete pre-RC must be formed on the replication origin (DePamphilis, 2003; Sun et al., 2002). For completion of the pre-RC, ORC1 is finally recruited to the ORC2-6 complex (Bell, 2002; DePamphilis, 2003). Although some reports showed that ORC1 as well as ORC2-6 interacted directly with LANA *in vitro* or *in vivo* (Lim et al., 2002; Verma et al., 2006), we could not confirm these results. Cell fractionation experiments (Fig. 1) showed that LANA and pre-RC components including ORC1, Cdc6, and Cdt1 were present together in the nuclear matrix fraction, which is a specialized part of nucleus in terms of resistance to DNaseI treatment and high salt condition, at the G1 phase, and at reduced levels in the S phase, even though only about 50–60% of the population was in the S phase. The ORC1 signal in the nuclear matrix in the G2/M phase could result from the remaining G1 phase population, or this fraction of ORC1 might function in the G2/M phase, consistent with the recent observation that not only ORC2-6 but also a portion of the ORC1 binds chromatin throughout the cell cycle (Laman et al., 2001; McNairn et al., 2005; Okuno et al., 2001). The release of the ORC1 from chromatin is believed to be modified by ubiquitination for its degradation (Li and DePamphilis, 2002), but it is also reported that the role of ubiquitination of ORC1 during the S-to-M transition does not result just in the destruction but also in its

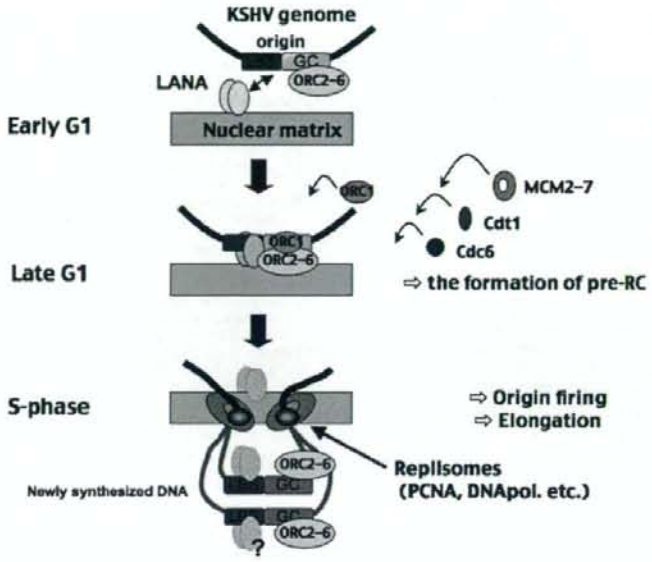


Fig. 5. A suggestive model for replication of the KSHV genome at the nuclear matrix. LANA associates with the nuclear matrix in itself and recruits the ori-P of KSHV genome to the nuclear matrix region by binding to the LBS. The complete pre-RC is formed on the nuclear matrix in the late G1 phase. In the S phase, the components of the pre-RC except for ORC2-6 are released from the origin by ubiquitination or phosphorylation. The replisome including PCNA, DNA polymerases, etc., is recruited to the nuclear matrix and kept there, and the origin DNA is unwound, and elongation is initiated. The replicated DNA is immediately released from the nuclear matrix region. The majority of LANA is thought to stay at the nuclear matrix from our analysis, though it is needed further to elucidate how and where replicated KSHV DNA is localized.

sequestration from the origin, to prevent its re-replication with the ORC-chromatin sites (Anachkova et al., 2005; Li and DePamphilis, 2002).

Experiments with BC3 cells expressing GFP-ORC1 suggested that some specialized ORC1 kept its intact form at nuclear matrix region. Such experiments also gave us a hint that nuclear matrix localization of LANA could be an important condition in terms of LANA-dependent viral replication in the latency, because it has been discussed about nuclear matrix localization of cellular replication machinery (Anachkova et al., 2005; Cook, 1999; Kitamura et al., 2006; Radichev et al., 2006).

Therefore, it was not surprising that we were unable to detect the interaction between LANA and ORC1 by immunoprecipitation assay, since pre-RC components such as ORC1, Cdc6, and Cdt1 localized to the nuclear matrix fraction, which is very insoluble with an ordinary buffer as mentioned and LANA was almost exclusively present at the nuclear matrix throughout the cell cycle. We showed that LANA and pre-RC components were present in the nuclear matrix fraction and the co-localization of LANA with pre-RC components and with NuMA, by IFA. It was reported that LANA and NuMA interacted each other and LANA might be recruited by NuMA in the nuclear matrix, though it is still unclear because gN-DBD, which retains the interacting region to NuMA, was fractionated into the chromatin fraction but not into the nuclear matrix fraction (Si et al., 2008). Although it is not clear either how much the fraction components vary cells from cells, NuMA might function to keep the virus genomes at a constant number. Different from Kaposi's sarcoma cell lines, PEL cell lines could have a special reason keeping the viral genome, which is partly because PEL requires KSHV function to be alive (Chen and Lagunoff, 2005; Ueda et al., 2006). Further investigation is required to confirm that these factors are directly associated with the nuclear matrix components.

On the other hand, some investigators have suggested that the origin of replication is associated with the nuclear matrix and that this association is cell-cycle dependent (Djeliova et al., 2001; Radichev et al., 2005), leading them to propose a model for formation of the pre-RC at the nuclear matrix (Anachkova et al., 2005). Our experiment confirmed these previous reports, and in case of KSHV genome, it was thought that LANA had an essential role for the recruitment of ori-P to the nuclear matrix since non-nuclear matrix-associated LANA mutants did not support the viral replication in a transient replication assay and the less effectiveness might be dependent on the loss of interaction with NuMA (Si et al., 2008), even though such mutants maintained the binding activity to the LBS (Garber et al., 2001; Hu et al., 2002; Kelley-Clarke et al., 2007; Komatsu et al., 2004).

ORC2-6 is known to bind to chromatin throughout the cell cycle, but ORC1 and MCMs are recruited to the origin only in the late G1 phase (Blow and Dutta, 2005; DePamphilis et al., 2006), and we showed that LANA constantly existed in the nuclear matrix region. Such data suggest that the cellular replication machinery for complete pre-RC formation probably functions for activation of the viral ori-P in the presence of LANA in a cell-cycle-dependent manner. In such a sense, an indirect action by LANA such as the interaction with histone acetyltransferase binding to ORC1 (HBO1) might be important to establish the ori-P activity (Stedman et al., 2004).

Furthermore, for the KSHV replication in latency, recent studies showed that the minimal replicator consists of LBS1/2 and a downstream 32-bp GC-rich segment (nt 539-610 in GenBank accession No. 75699) (Hu and Renne, 2005). Our experiment further confirmed that one of the two LANA-binding sites and the 32-bp GC-rich downstream segment were required and sufficient for the replication, independent of their orientation, though the orientation might affect the efficiency (our personal communication and the similar result by Hu et al. [page 29 in the Abstract of "the

10th International Workshop on Kaposi's Sarcoma Associated Herpesvirus (KSHV) and Related Agents", August 1-5, Portland, OR]). Neither the LBS-binding sites nor the GC-rich segment alone was sufficient for the viral ori-P activity. The requirement of the 32-bp GC-rich segment remains to be elucidated; if LANA recruits ORCs by its binding activity with them, the LBS-binding sites should be sufficient to initiate replication, because the DNA-binding activity of ORCs is not sequence specific, although it has a preferred sequence (Vashee et al., 2003), and the GC-rich segment is rather unusual as a replicator. Thus, further investigation is needed to elucidate how the LBS and 32-bp GC-rich downstream segment function in viral replication in latency.

It has been demonstrated by live-cell imaging that DNA replication of chromosomal loci occurs at replication factories where the bulk of DNA synthesis takes place (Leonhardt et al., 2000). Such a replisome in the S phase is associated with replication factories, and the replicated DNA separates from there (Cook, 1999). We demonstrated that not only LANA and the pre-RC, but also the TR region of the KSHV genome preferentially localized to the nuclear matrix fraction, especially in the G1 phase. The DNA in the nuclear matrix fraction was resistant to DNase I treatment and also to high salt condition, thus the detection of the TR region in this assay was very suggestive of a strong association of ori-P with a nuclear matrix component occurring in the G1 phase.

Taken together, these experiments suggest that a viral latent protein, LANA, recruits the viral latent origin to the nuclear matrix through its binding activity to the LBS, which leads to the formation of the complete pre-RC on the GC-rich segment to initiate viral replication in a cell-cycle-dependent manner (Fig. 5). This process could not be necessarily achieved by the direct interaction between LANA and ORCs, and at least, such a recruitment factor for ORCs have not been identified in case of host genome replication system. Needless to say, further experiments will be needed to show whether the GC-rich segment is in fact required for the pre-RC placement. Moreover, elucidation of the fundamental mechanism of KSHV genome replication in latency may shed light on how the mammalian replication origin is determined.

Acknowledgements

This work was supported by grants from the Japan Science and Technology Agency PRESTO (No. 200154023) and the Japan Ministry of Education, Culture, Sports and Science and Technology (No. 17050016) to K.U. We thank Dr. Shuhei Sakakibara for his technical assistance.

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HCVと肝発癌
——HCVの感染性ウイルス粒子産生と細胞内環境
Hepatocellular carcinogenesis caused by HCV infection

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◎HCV感染による肝癌発症には，長期にわたる慢性肝炎による継続的な細胞増殖がその原因のひとつとしてあげられる．HCV感染細胞の癌化には，HCV蛋白質が感染した細胞に対して何らかの影響を与えると考えられる．HCV蛋白質のなかではコアが，現時点ではもっとも肝発癌と関連した蛋白質と考えられている．最近，培養細胞を用いた組換え体HCV産生実験系が確立され，著者らはこの実験系を用いてHCVのウイルス粒子産生機構について解析した．その結果，感染性HCV粒子の産生には細胞内の脂肪滴が重要な役割をもつことを明らかにした．このようなコアを発現している細胞では細胞内の脂肪滴の量が上昇し，中性脂肪量が上昇していることが確認された．このことはC型肝炎肝組織中における脂肪の蓄積が感染性ウイルス粒子産生と密接に関連する可能性を示している．近年，脂肪滴の多機能性が示されているため，コアの局在化が脂肪滴の機能修飾を誘導し，細胞の機能を変化させる可能性が考えられる．

Key word：C型肝炎ウイルス，感染性ウイルス粒子産生，脂肪滴，コア蛋白質

1989年にアメリカのベンチャー企業であるカイロン社によって，それまで非A非B型肝炎ウイルスとよばれていたウイルスの遺伝子がはじめてクローニングされ，C型肝炎ウイルス(hepatitis C virus：HCV)と命名された[1]．これまでの疫学的研究から現在，世界保健機構(WHO)によって世界人口の3%以上がすでにこのウイルスに感染していると報告されている．この感染者人口の多さに加えて，このウイルスに感染することによっては長期にわたる慢性肝炎が引き起こされ，それは20〜30年後に肝硬変から肝癌へと進行する可能性が高いことから，このウイルスは人類に対する大きな脅威のひとつであると認識されている．

残念ながらHCV感染による肝癌発症のメカニズムの詳細についてはいまだに明らかになっているわけではないが，多くの研究者の努力によりこれまでにさまざまな重要な知見が蓄積されてきている．本稿ではウイルス学的な見地から，HCV感染による肝発癌機構について考察したいと思う．

HCV感染と肝発癌

HCVの遺伝子構造の概要が明らかとなり，このウイルスがフラビウイルス科に分類される一本鎖RNAウイルスであることがわかった．このウイルスの近縁種には日本脳炎ウイルスや黄熱病ウイルスなどがあり，これまでにみつかっている癌ウイルスつまりB型肝炎ウイルスなどのDNAウイルスや，ヒトT細胞白血病ウイルスのようなレトロウイルスとはまったく異なるウイルスであった．

発癌ウイルスとして知られるこれらのウイルスに共通する特徴のひとつは，これらのウイルス遺伝子全体あるいはその一部が，感染した宿主細胞の染色体に組み込まれる，あるいはEpstein-Barr virusのようにエピソームとして細胞内に存在するということである．したがって，これらのウイルスによって癌化した細胞の場合，染色体上にウイルス感染の証拠としてウイルス遺伝子全体あるいは一部が検出されるか，細胞内にウイルスゲノ

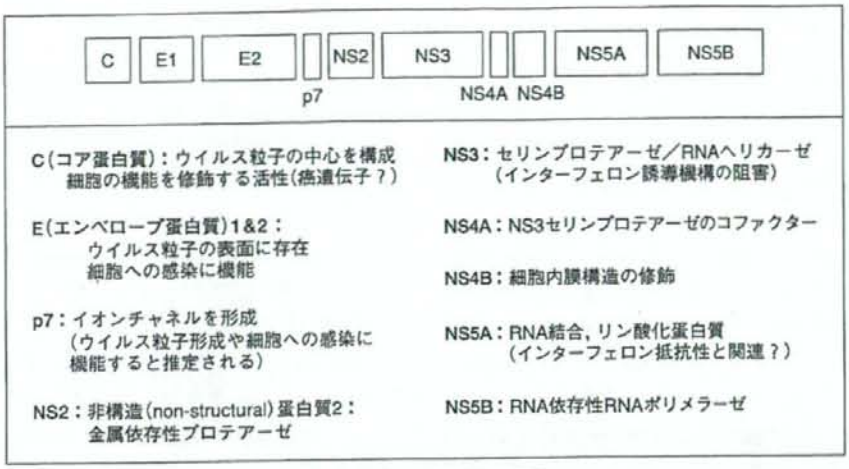

図 1　HCV蛋白質とその機能

ムが存在する．しかし，HCV を含むフラビウイルスの生活環にはその遺伝子が DNA となるステップは存在せず，実際に HCV 感染と密接に関連する肝癌組織のなかに HCV 由来 DNA はこれまでのところ見出されていない．そして肝癌組織から HCV の感染増殖を維持している細胞株を樹立することにも成功していない．また，肝癌組織のなかの HCV の RNA 遺伝子量ですら非癌部の組織よりもむしろ少ない．

このことはすくなくとも，HCV が感染した肝のなかで，癌化する細胞がどのような細胞に由来するのか正確にはわかっていないということを意味している．つまり HCV 感染と肝癌発症は密接に関連することは明らかであるにもかかわらず，癌化した細胞が実際に HCV の感染を受けたことがあるのかどうかについてさえ，なんら確証はないことになる．そこで，HCV 感染による肝癌発症には，①HCV に感染した肝細胞の癌化と，②非感染細胞の癌化，という 2 つの可能性を考える必要があるように思われる．

HCV蛋白質による細胞機能の変化

HCV 感染による肝発癌の場合，前述したようにウイルス遺伝子断片が感染細胞の染色体に挿入されることにより細胞の遺伝子発現異常が引き起こされている可能性はきわめて低い．したがって，HCV 感染細胞が癌化すると考えた場合，その遺伝子産物つまり HCV 蛋白質が感染した細胞に対して何らかの影響を与える可能性が考えられる．このことから，その細胞に対する影響を同定するために，培養細胞やマウスのような実験動物に各種 HCV 蛋白質を発現させて，その結果どのような変化が現れるのかを解析するという研究方法が用いられている．また，各 HCV 蛋白質と相互作用する細胞因子の候補分子を同定し，分子レベルからこの問題にアプローチする方法も用いられている．

図 1 に HCV 遺伝子とその産物の概略を示した．HCV は一本鎖 RNA ウイルスであり，その増殖は細胞質で行われていると考えられる．約 9,500 ヌクレオチド長の RNA ゲノムの大部分は，1 つの蛋白質読み枠(open reading frame：ORF)で占められている．ウイルス蛋白質はすべてここにコードされており，主要なウイルス蛋白質はその ORF から翻訳される前駆体ポリ蛋白質から，宿主側あるいはそのポリ蛋白質に存在するプロテアーゼ活性によってプロセッシングされて産生されてくる[2]．この ORF の 5'末端側からウイルス粒子の中心を形成するコア(C)，ウイルス粒子表面に存在するエンベロープ 1 そして 2(E1，E2)といったウイルス粒子を構成する構造蛋白質群，その C 末端側からは，p7 とよばれるイオンチャネルを形成する分子，さらにゲノム複製などに関与すると考えられる 6 種の非構造(nonstructural：NS)蛋白質

(NS2〜NS5B)が産生されることがわかっている(図1).

そのいくつかの蛋白質について培養細胞を用いたその形質転換やアポトーシスに対する影響の解析，そしてその効果を裏打ちする細胞内シグナルの修飾に関する研究が行われてきている[3]．しかし，HCV 蛋白質に関するこうした研究の結果には相反するさまざまな報告がなされ，一定の見解が得られていない場合があり，HCV 蛋白質の感染細胞に対する機能を明らかにするうえで大きな問題となっている．こうした問題は，HCV の多様性，つまり用いた HCV 蛋白質の一次構造上の多様性やそれぞれの実験で使用した細胞種あるいは HCV 蛋白質の発現方法や発現量，生理活性の検出系といった実験条件の相違に起因するのかもしれない．今後，HCV の感染増殖過程のどのような場面でこれまでに得られた結果が再現されるのか，正常な肝細胞を用いて HCV の生活環を再現する実験系を構築し，研究を進めていく必要があると思われる．

HCVコア蛋白質による形質転換

HCV 蛋白質のなかでコアが現時点でもっとも肝発癌と関連した蛋白質と考えられている．まず，コアは癌遺伝子のひとつである活性型 Ras と同時に発現されることによって，ラット胎仔線維芽細胞を形質転換するということが最初に報告された[4]．このことは形質転換実験でよく用いられるマウス BALB/c 3T3 A31-1-1 細胞を用いて著者らの研究室でも再現された[5]．さらに，コアを発現するトランスジェニックマウス(コア Tg マウス)には長期間の飼育の後に肝発癌するものが出てくることが報告された[6]．このコア Tg マウスの詳細な解析は本誌他稿に紹介されているので参照されたい．

これらの結果はすべて，コアによる細胞の形質転換能が比較的弱いものであることを示唆している．つまりコア単独では細胞の形質転換を誘導できず，コア Tg マウスでもすべての系統における早期の肝発癌が観察されないからである．それではコアは何をしているのか．このコア Tg マウス肝組織には，コントロールマウスに比較して 1.8 倍の過酸化脂質が存在することが検出されたが[7]，培養細胞においてコアを発現させた場合でも同様に過酸化脂質産生の上昇や抗酸化反応因子の遺伝子発現誘導が観察されていることから，コアの発現により遺伝子傷害性をもつ活性酸素の産生が上昇する可能性が示唆されている[8]．このどちらの場合でもミトコンドリアの損傷が認められていることから，コア発現による活性酸素の産生にはミトコンドリアの関与が示唆されている．ここでコアの細胞内局在は主として小胞体や脂肪滴であり，ミトコンドリアに局在するコアは量的に限られることから，どのようなメカニズムでコアがミトコンドリアに障害を引き起こしているのかさらに慎重に解析を進める必要があると思われる．

HCVコア蛋白質と脂肪滴

最近，培養細胞を用いた組換え体 HCV の産生実験系が確立された[9]．これは劇症肝炎患者由来の HCV 遺伝子 JFH-1(遺伝子型 2a)を試験管内で合成し，これを肝癌由来細胞 HuH-7 細胞に導入すると，その培養上清中に組換え体ウイルスが産生されるものである．この組換え体ウイルスはこの HuH-7 細胞からクローン化された HuH-7.5 細胞や HuH-7.5.1 細胞によく感染増殖し，ふたたびその上清に感染性 HCV を産生することができるのである．

著者らはこの実験系を用いて HCV のウイルス粒子産生機構について解析し，感染性 HCV 粒子の産生には細胞内の脂肪滴(「サイドメモ1」参照)が重要な役割をもつことを明らかにした(図2)[10]．この感染性 HCV 粒子を産生している細胞では脂

サイドメモ1 脂肪滴

小胞体膜に由来する脂質単層膜で包まれた構造をもつ．細胞内小器官のひとつである．トリアシルグリセロールやコレステロールエステルなどの中性脂肪を含むことが知られている．これまでこれら脂肪の蓄積や供給にかかわることが知られていたが，近年，種々の細胞内小器官への脂質輸送や蛋白質の繋留や分解の場として機能することが示されてきている．

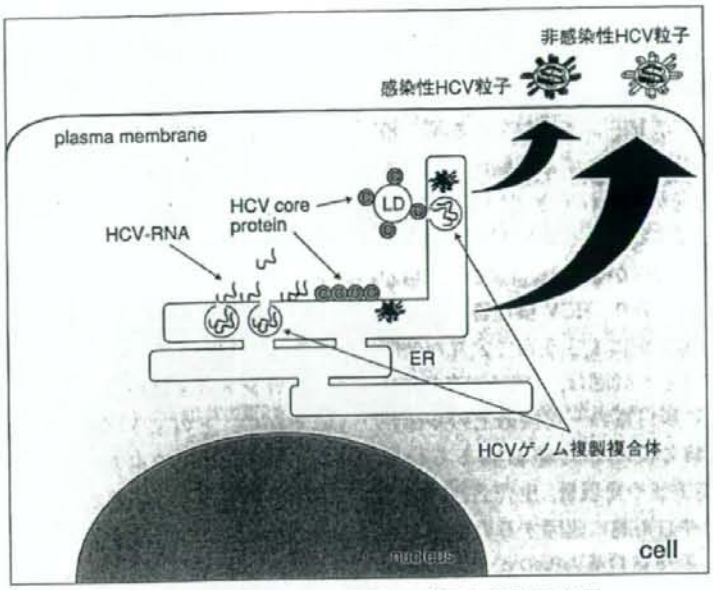

図 2　脂肪滴が関与する感染性HCV粒子産生機構の概要
ER：endoplasmic reticulum(小胞体)，LD：lipid droplet(脂肪滴)．

肪滴膜上にコアが局在化しており，その周囲の膜構造にエンベロープ蛋白質や複製活性のあるHCV遺伝子複製複合体を含むNS蛋白質群が存在している．電子顕微鏡で観察するとウイルス粒子はその脂肪滴の周囲にある膜構造のなかに認められる．上清に含まれるHCV粒子には浮遊密度1.12をピークとする感染性粒子と1.15をピークとする非感染性粒子とが存在し，その双方にコアと遺伝子RNAが含まれていることがわかった．しかもコアや遺伝子RNA量でみるかぎり非感染性粒子に比べて感染粒子の量はきわめて少ないことがわかった．組換え体HCV遺伝子において脂肪滴に局在化しない点突然変異をコアに導入すると，HCV遺伝子の複製は正常であるが，他のHCV蛋白質は脂肪滴周囲には存在せず，感染性ウイルスの産生は認められない．また，同様にNS5Aにコアと相互作用しないアミノ酸変異を導入した場合には，コアは脂肪滴上に存在しHCV遺伝子複製は認められるにもかかわらず，他のHCV蛋白質は脂肪滴周囲には存在せず，感染性ウイルスの産生はほとんど認められない．ただし，このNS5A変異型JFH-1の場合でも培養上清にはHCVコアとHCV RNAを含む非感染性粒子の産生は認められるため，この系は脂肪滴とは重要な関係を有しないものであると考えられる．このような感染性ウイルス産生系においてコアを発現している細胞で可視化できる脂肪滴の数量が上昇していた(図3)．

そして感染性粒子産生細胞では，中性脂肪量が上昇していることが認められた(図4)．このことは，上述したコアTgマウスにおいて全例脂肪肝が認められ，コアの発現により肝に脂肪が蓄積することや慢性C型肝炎患者の肝に脂肪肝がよく認められることと一致すると思われる．つまり，HCV感染肝細胞中における脂肪の蓄積は感染性ウイルス粒子産生のために必然的なものなのかもしれない．

近年，脂肪滴の機能が細胞内における中性脂肪の貯蔵であるだけでなく，細胞内膜系や種々のオルガネラへの脂質輸送や蛋白質の繋留や分解の場として機能することが示されてきている[11,12]．つまり脂肪滴にコアが局在化することがこうした脂肪滴の機能障害が活性化を引き起こし，その結果，細胞内のシグナル経路の修飾や上述したミトコンドリア損傷に関係する可能性もあるかもしれない．

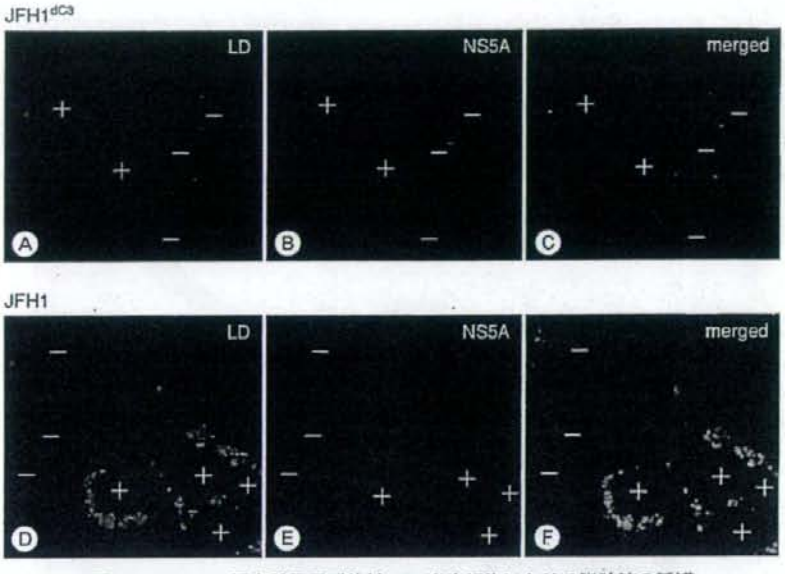

図 3　HCVコア蛋白質発現感染性HCV産生細胞における脂肪滴の誘導
コア領域を欠損した JFH1dC3 遺伝子 RNA あるいは野生型同等以上に感染性の高いウイルスをつくる JFH1E2FL RNA を導入した Huh-7 細胞の脂肪滴の検出．RNA が導入された細胞は HCV NS5A の発現で確認される．

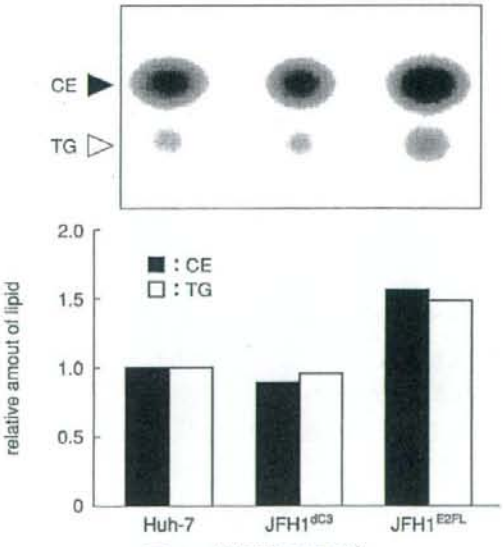

図 4　中性脂肪の蓄積[10]
未処理 Huh-7 細胞と図 3 で用いた RNA を導入した細胞内の中性脂肪を薄層クロマトグラフィーで分離定量した．CE：コレステロールエステル，TG：トリアシルグリセロール．

肝炎と肝発癌

HCV 感染による肝癌の発症は 20〜30 年という長い慢性肝炎を経過した後に起こることが知られている．ウイルス性肝炎は一般的にウイルスが感染した細胞を宿主の免疫系が攻撃し，これを破壊するという生体反応であると考えられている．肝ではこうして排除された肝細胞は再生によって補われる．したがって，慢性 C 型肝炎で長期にわたって継続的な肝細胞の破壊と再生が繰り返し行われることは，肝細胞の増殖活性化と同じ意味をもつことになるのかもしれない．

初代培養細胞の形質転換の前段階に細胞の不死化(「サイドメモ 2」参照)という継続的な細胞増殖の段階が存在するように，継続的な細胞増殖は遺伝子変異の蓄積を引き起こす原因と考えられている．さらに，肝細胞は恒常的に免疫系細胞群からの種々の炎症性サイトカインや遺伝子変異を引き起こす活性酸素に曝されることになると思われる．つまり HCV 感染による肝発癌の原因のひとつが慢性肝炎そのものである可能性が考えられる．このことは，かならずしも癌化する細胞に HCV が感染する必要がないことを意味するかも

しれない．

しかし，慢性肝炎のなかでも自己免疫性肝炎症例の場合，肝硬変へと進行するが，肝癌の発症はウイルス性肝炎ほどではないことが知られている．自己免疫性肝炎とC型慢性肝炎の肝細胞に及ぼす影響の質的な相違は明らかではないが，すくなくともHCV感染による慢性肝炎が肝発癌への重要な要因となっており，HCVの何らかの要因がさらにその進行を加速する可能性は否定できないと考えられる．

おわりに

HCV感染による肝発癌には長期の慢性肝炎が重要な原因となっていると思われる．HCV感染が果たす役割は肝炎を誘導する以外にも，感染した細胞自体に癌化を促す作用がある可能性が考えられる．これには，HCVの感染が細胞の癌化に必要ないくつかのステップにかかわる宿主細胞の遺伝子変化を誘導することによって正常細胞を癌化させるというメカニズムが考えられている．

今回，著者らの研究から，HCVの感染性粒子産生機構そのものがヒトの肝細胞のとくに脂肪滴に変化を及ぼすことが明らかとなった．近年，脂肪滴の機能が単に中性脂肪の蓄積にとどまらず，さまざまな生命現象と関連することが示されてきている．このことから，たとえばHCVコア蛋白質の脂肪滴への局在化を抑制する方法が確立すれば，HCVの感染性粒子産生を阻害するのみならず，脂肪滴を介した細胞への影響を抑えることになるため，HCVによる肝発癌に対して効果の高い予防戦略となる可能性があると考えられる．

サイドメモ2　不死化

正常な体細胞は通常寿命をもっており，分裂を繰り返すと死ぬ場合がほとんどである．このことは細胞が分裂限界を有するということを意味するが，不死化とは細胞の分裂限界がなくなり，半永久的に分裂可能な状態になることである．癌化または形質転換とは異なり，無軌道に分裂するわけではなく，分裂には増殖因子刺激が必要であったり細胞密度が上昇しすぎると分裂を停止するなど，ある一定の秩序をもった増殖を行う．通常細胞の癌化への全段階と考えられている．

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