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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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).

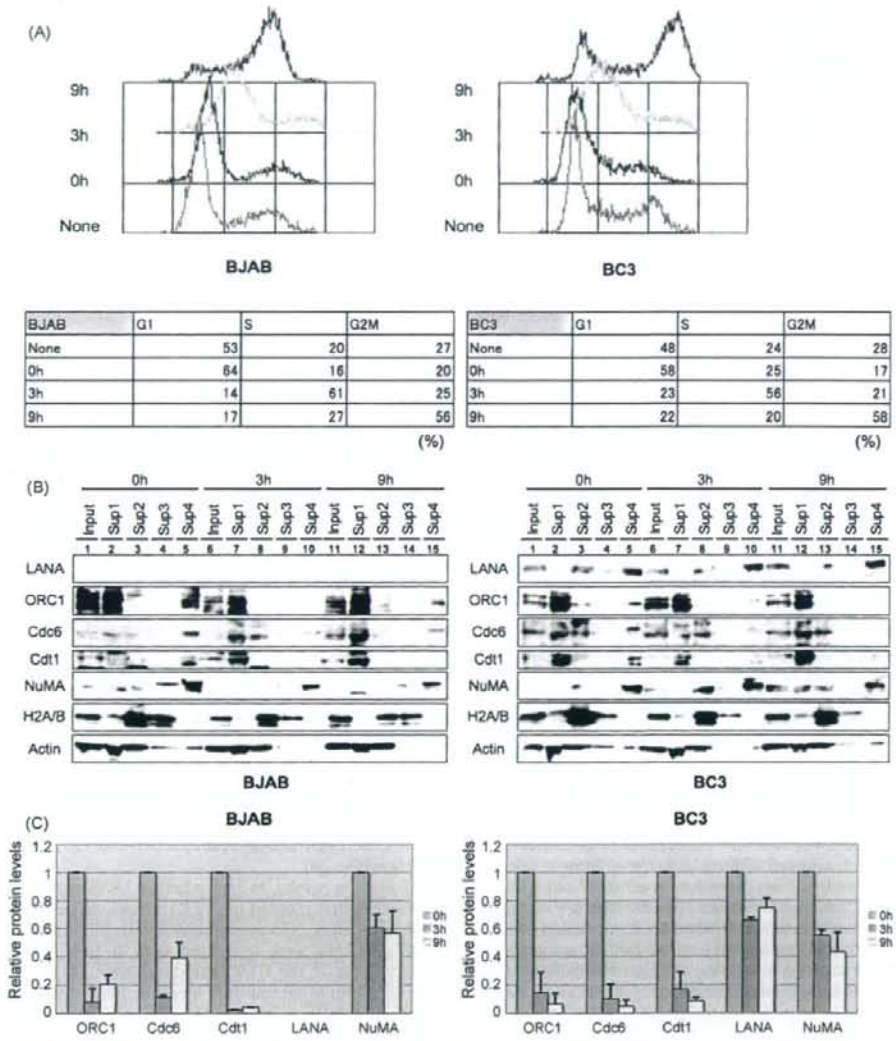
3. Results

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	16	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

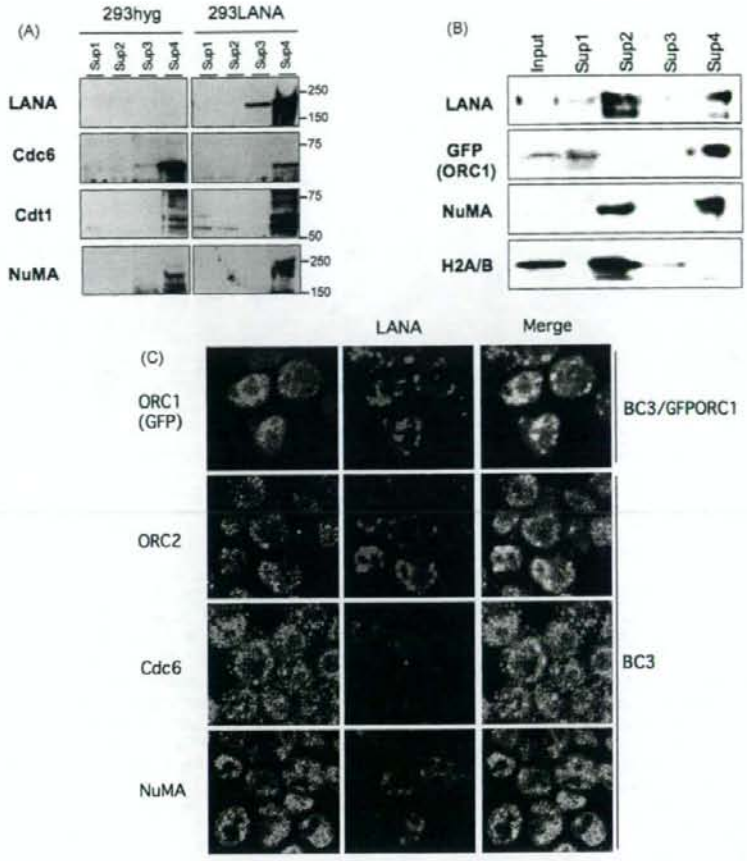


Fig. 2. LANA self-associates with the nuclear matrix fraction and colocalizes with host pre-RC components. (A) One million 293hyg or 293LANA cells were harvested and fractionated. Ten percent of each fraction was separated on 10% SDS-PAGE and subjected to Western blotting analysis. (B) One million GFP-ORC1/BC3 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

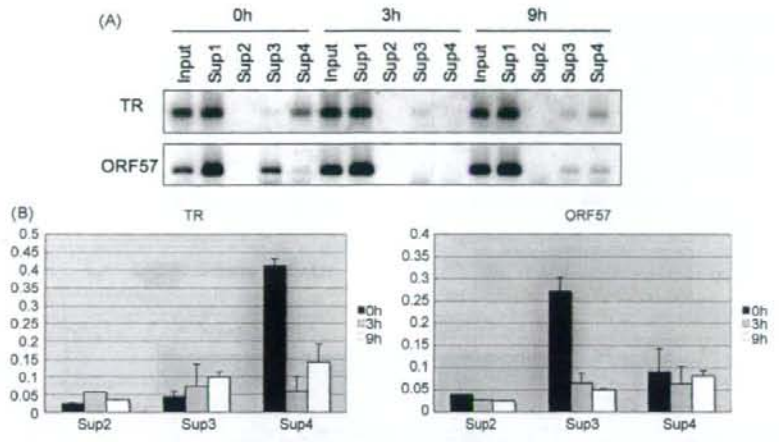


Fig. 3. The TR region (ori-P) is present in the nuclear matrix in a cell-cycle-dependent manner. DNA from fractions of the BC3 cells prepared in Fig. 1C was extracted as described in Section 2. The amount of DNA was measured by spectrophotometer (DU640, Beckman) and was adjusted to 10 ng/μl. Input DNA 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

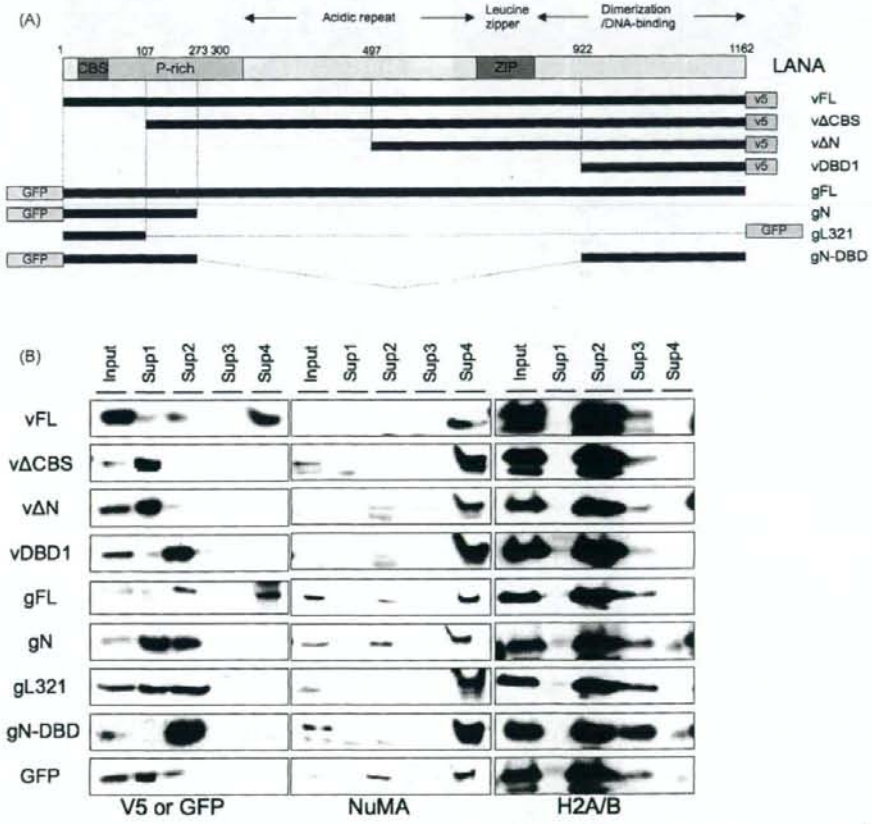


Fig. 4. Nuclear localization of LANA is required for ori-P mediated viral replication. (A) Schematic presentation of LANA expression constructs. Two full-length LANA expression vector was constructed. One was tagged at the C-terminus with a V5 epitope (vFL) and another was at the N-terminus with a GFP 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.

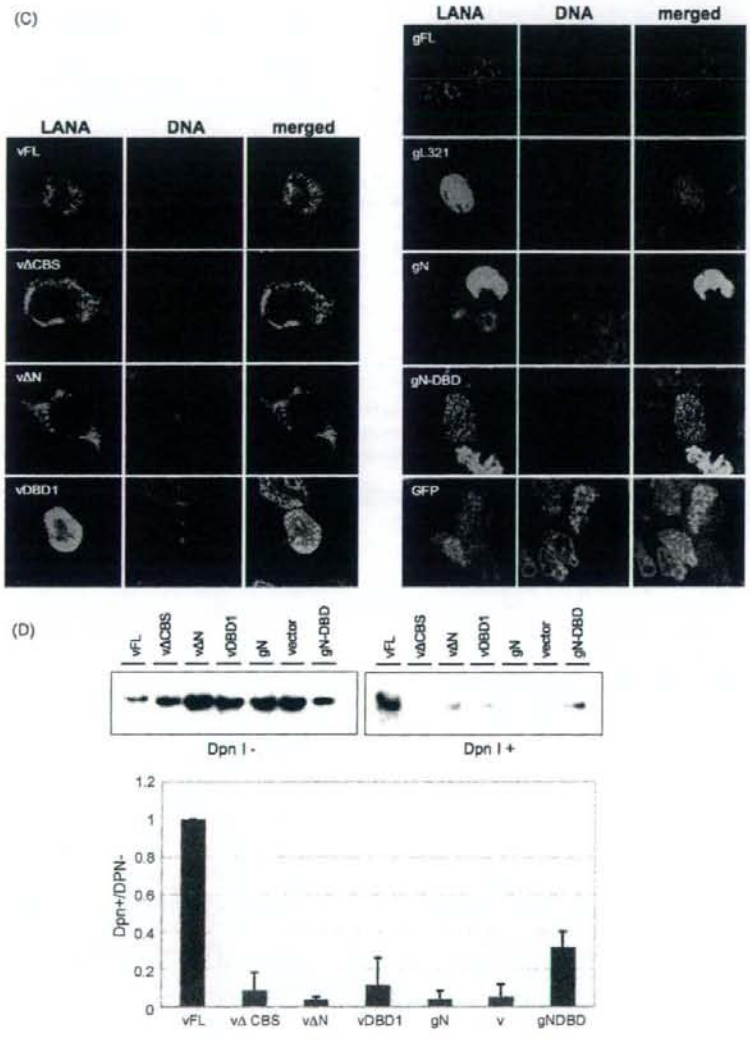


Fig. 4. *(Continued).*

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

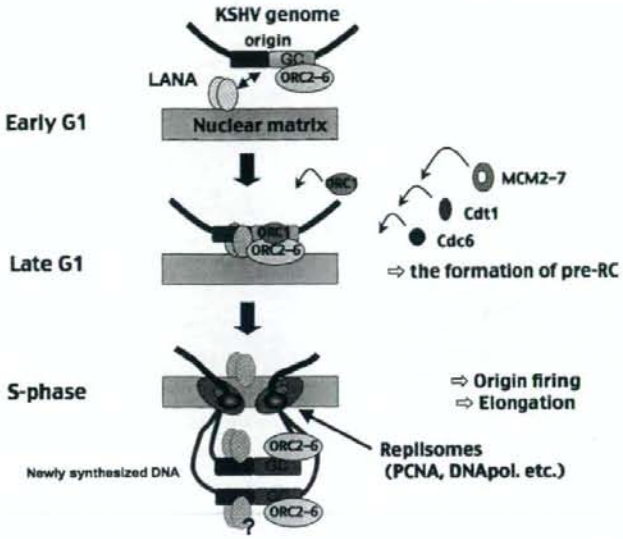


Fig. 5. A suggestive model for replication of the KSHV genome at the nuclear matrix. LANA associates with the nuclear matrix in itself and recruits the ori-P of KSHV genome to the nuclear matrix region by binding to the LBS. The complete pre-RC is formed on the nuclear matrix in the late G1 phase. In 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.

References

Anachkova, B., Djeliova, V., Russev, G., 2005. Nuclear matrix support of DNA replication. J. Cell. Biochem. 96 (5), 951–961.
Ballestas, M.E., Chatis, P.A., Kaye, K.M., 1999. Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. Science 284 (5414), 641–644.
Bechtel, J.T., Liang, Y., Hvidding, J., Ganem, D., 2003. Host range of Kaposi's sarcoma-associated herpesvirus in cultured cells. J. Virol. 77 (11), 6474–6481.
Bechtel, J.T., Winant, R.C., Ganem, D., 2005. Host and viral proteins in the virion of Kaposi's sarcoma-associated herpesvirus. J. Virol. 79 (8), 4952–4964.
Belgrader, P., Siegel, A.J., Berezney, R., 1991. A comprehensive study on the isolation and characterization of the HeLa S3 nuclear matrix. J. Cell. Sci. 98 (Pt 3), 281–291.
Bell, S.P., 2002. The origin recognition complex: from simple origins to complex functions. Genes Dev. 16 (6), 659–672.
Berezney, R., Mortillaro, M.J., Ma, H., Wei, X., Samarabandu, J., 1995. The nuclear matrix: a structural milieu for genomic function. Int. Rev. Cytol. 162A, 1–65.
Blow, J.J., Dutta, A., 2005. Preventing re-replication of chromosomal DNA. Nat. Rev. Mol. Cell Biol. 6 (6), 476–486.
Boshoff, C., Weiss, R.A., 2001. Epidemiology and pathogenesis of Kaposi's sarcoma-associated herpesvirus. Philos. Trans. R. Soc. Lond. B: Biol. Sci. 356 (1408), 517–534.
Cesarman, E., Chang, Y., Moore, P.S., Said, J.W., Knowles, D.M., 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N. Engl. J. Med. 332 (18), 1186–1191.
Chang, Y., Moore, P.S., 1996. Kaposi's sarcoma (KS)-associated herpesvirus and its role in KS. Infect. Agents Dis. 5 (4), 215–222.
Chang, Y., Cesarman, E., Pessin, M.S., Lee, F., Culpepper, J., Knowles, D.M., Moore, P.S., 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 266 (5192), 1865–1869.
Chaudhuri, B., Xu, H., Todorov, I., Dutta, A., Yates, J.L., 2001. Human DNA replication initiation factors, ORC and MCM, associate with oriP of Epstein–Barr virus. Proc. Natl. Acad. Sci. U.S.A. 98 (18), 10085–10089.
Chen, L., Lagunoff, M., 2005. Establishment and maintenance of Kaposi's sarcoma-associated herpesvirus latency in B cells. J. Virol. 79 (22), 14383–14391.
Cook, P.R., 1999. The organization of replication and transcription. Science 284 (5421), 1790–1795.
Cook, P.R., Brazell, I.A., 1975. Supercoils in human DNA. J. Cell. Sci. 19 (2), 261–279.
Cotter 2nd, M.A., Robertson, E.S., 1999. The latency-associated nuclear antigen tethers the Kaposi's sarcoma-associated herpesvirus genome to host chromosomes in body cavity-based lymphoma cells. Virology 264 (2), 254–264.
DePamphilis, M.L., 2003. The 'ORC cycle': a novel pathway for regulating eukaryotic DNA replication. Gene 310, 1–15.
DePamphilis, M.L., Blow, J.J., Ghosh, S., Saha, T., Noguchi, K., Vassilev, A., 2006. Regulating the licensing of DNA replication origins in metazoa. Curr. Opin. Cell Biol. 18 (3), 231–239.
Dhar, S.K., Yoshida, K., Machida, Y., Khaira, P., Chaudhuri, B., Wohlschlegel, J.A., Leffak, M., Yates, J., Dutta, A., 2001. Replication from oriP of Epstein–Barr virus requires human ORC and is inhibited by geminin. Cell 106 (3), 287–296.
Djeliova, V., Russev, G., Anachkova, B., 2001. Dynamics of association of origins of DNA replication with the nuclear matrix during the cell cycle. Nucl. Acids Res. 29 (15), 3181–3187.
Fejer, G., Medveczky, M.M., Horvath, E., Lane, B., Chang, Y., Medveczky, P.G., 2003. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus interacts preferentially with the terminal repeats of the genome in vivo and this complex is sufficient for episomal DNA replication. J. Gen. Virol. 84 (Pt 6), 1451–1462.
Garber, A.C., Shu, M.A., Hu, J., Renne, R., 2001. DNA binding and modulation of gene expression by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. J. Virol. 75 (17), 7882–7892.
Gehmlich, K., Haren, L., Merdes, A., 2004. Cyclin B degradation leads to NuMA release from dynein/dynactin and from spindle poles. EMBO Rep. 5 (1), 97–103.
Grundhoff, A., Ganem, D., 2004. Inefficient establishment of KSHV latency suggests an additional role for continued lytic replication in Kaposi sarcoma pathogenesis. J. Clin. Invest. 113 (1), 124–136.
Hu, J., Renne, R., 2005. Characterization of the minimal replicator of Kaposi's sarcoma-associated herpesvirus latent origin. J. Virol. 79 (4), 2637–2642.
Hu, J., Garber, A.C., Renne, R., 2002. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus supports latent DNA replication in dividing cells. J. Virol. 76 (22), 11677–11687.
Jackson, D.A., Cook, P.R., 1995. The structural basis of nuclear function. Int. Rev. Cytol. 162A, 125–149.
Jenke, A.C.W., Stehle, I.M., Herrmann, F., Eisenberger, T., Baiker, A., Bode, J., Faclelmayer, F.O., Lipps, H.J., 2004. Nuclear scaffold/matrix attached region modules linked to a transcriptption unit are sufficient for replication and maintenance of a mammalian genome. Proc. Natl. Acad. Sci. U.S.A. 101 (31), 11322–11327.
Kelley-Clarke, B., Ballestas, M.E., Komatsu, T., Kaye, K.M., 2007. Kaposi's sarcoma herpesvirus C-terminal LANA concentrates at pericentromeric and pericentromeric regions of a subset of mitotic chromosomes. Virology 357 (2), 149–157.
Kitamura, E., Blow, J.J., Tanaka, T.U., 2006. Live-cell imaging reveals replication of individual replicons in eukaryotic replication factories. Cell 125 (7), 1297–1308.
Komatsu, T., Ballestas, M.E., Barbera, A.J., Kelley-Clarke, B., Kaye, K.M., 2004. KSHV LANA1 binds DNA as an oligomer and residues N-terminal to the oligomerization domain are essential for DNA binding, replication, and episome persistence. Virology 319 (2), 225–236.
Lagunoff, M., Bechtel, J., Venetsanakos, E., Roy, A.M., Abbey, N., Herndier, B., McMahon, M., Ganem, D., 2002. De novo infection and serial transmission of Kaposi's sarcoma-associated herpesvirus in cultured endothelial cells. J. Virol. 76 (5), 2440–2448.
Laman, H., Peters, G., Jones, N., 2001. Cyclin-mediated export of human Orc1. Exp. Cell Res. 271 (2), 230–237.
Leonhardt, H., Rahn, H.P., Weinzierl, P., Sporbert, A., Cremer, T., Zink, D., Cardoso, M.C., 2000. Dynamics of DNA replication factories in living cells. J. Cell Biol. 149 (2), 271–280.
Li, C.J., DePamphilis, M.L., 2002. Mammalian Orc1 protein is selectively released from chromatin and ubiquitinated during the S-to-M transition in the cell division cycle. Mol. Cell Biol. 22 (1), 105–116.
Lim, C., Sohn, H., Lee, D., Gwack, Y., Choe, J., 2002. Functional dissection of latency-associated nuclear antigen 1 of Kaposi's sarcoma-associated herpesvirus involved in latent DNA replication and transcription of terminal repeats of the viral genome. J. Virol. 76 (20), 10320–10331.
Mattia, E., Ceridono, M., Chichiarelli, S., D'Erme, M., 1999. Interactions of Epstein–Barr virus origins of replication with nuclear matrix in the latent and in the lytic phases of viral infection. Virology 262 (1), 9–17.

McNairn, A.J., Okuno, Y., Misteli, T., Gilbert, D.M., 2005. Chinese hamster ORC subunits dynamically associate with chromatin throughout the cell-cycle. Exp. Cell Res. 308 (2), 345–356.
Mika, S., Rost, B., 2005. NMPdb: database of nuclear matrix proteins. Nucl. Acids Res. 33, D160–D163 (Database Issue).
Nakayasu, H., Berezney, R., 1989. Mapping replicational sites in the eucaryotic cell nucleus. J. Cell Biol. 108 (1), 1–11.
Neri, L.M., Mazzotti, G., Capitani, S., Maraldi, N.M., Cinti, C., Baldini, N., Rana, R., Martelli, A.M., 1992. Nuclear matrix-bound replicational sites detected in situ by 5-bromodeoxyuridine. Histochemistry 98 (1), 19–32.
Numazaki, K., Chiba, S., Aoki, K., Suzuki, K., Ohno, S., 1998. Human herpesvirus 8 variants. Lancet 351 (9103), 680.
Ohsaki, E., Ueda, K., Sakakibara, S., Do, E., Yada, K., Yamanishi, K., 2004. Poly (ADP-ribose) polymerase 1 binds to Kaposi's sarcoma-associated herpesvirus (KSHV) terminal repeat sequence and modulates KSHV replication in latency. J. Virol. 78 (18), 9936–9946.
Ohta, S., Tatsumi, Y., Fujita, M., Tsurimoto, T., Obuse, C., 2003. The ORC1 cycle in human cells. II. Dynamic changes in the human ORC complex during the cell cycle. J. Biol. Chem. 278 (42), 41535–41540.
Okuno, Y., McNairn, A.J., den Elzen, N., Pines, J., Gilbert, D.M., 2001. Stability, chromatin association and functional activity of mammalian pre-replication complex proteins during the cell cycle. EMBO J. 20 (15), 4263–4277.
Pardoll, D.M., Vogelstein, B., Coffey, D.S., 1980. A fixed site of DNA replication in eucaryotic cells. Cell 19 (2), 527–536.
Payrastre, B., Nievers, M., Boonstra, J., Breton, M., Verkleij, A.J., Van Bergen en Henegouwen, P.M., 1992. A differential location of phosphoinositide kinases, diacylglycerol kinase, and phospholipase C in the nuclear matrix. J. Biol. Chem. 267 (8), 5078–5084.
Radichev, I., Parashkevova, A., Anachkova, B., 2005. Initiation of DNA replication at a nuclear matrix-attached chromatin fraction. J. Cell. Physiol. 203 (1), 71–77.
Radichev, I., Kwon, S.W., Zhao, Y., DePamphilis, M.L., Vassilev, A., 2006. Genetic analysis of human Orc2 reveals specific domains that are required in vivo for assembly and nuclear localization of the origin recognition complex. J. Biol. Chem. 281 (32), 23264–23273.
Reyes, J.C., Muchardt, C., Yaniv, M., 1997. Components of the human SWI/SNF complex are enriched in active chromatin and are associated with the nuclear matrix. J. Cell Biol. 137 (2), 263–274.
Ritzi, M., Tillack, K., Gerhardt, J., Ott, E., Humme, S., Kremmer, E., Hammerschmidt, W., Schepers, A., 2003. Complex protein-DNA dynamics at the latent origin of DNA replication of Epstein–Barr virus. J. Cell Sci. 116 (Pt 19), 3971–3984.
Sakakibara, S., Ueda, K., Nishimura, K., Do, E., Ohsaki, E., Okuno, T., Yamanishi, K., 2004. Accumulation of heterochromatin components on the terminal repeat sequence of Kaposi's sarcoma-associated herpesvirus mediated by the latency-associated nuclear antigen. J. Virol. 78 (14), 7299–7310.
Schepers, A., Ritzi, M., Bousset, K., Kremmer, E., Yates, J.L., Harwood, J., Diffley, J.F., Hammerschmidt, W., 2001. Human origin recognition complex binds to the region of the latent origin of DNA replication of Epstein–Barr virus. EMBO J. 20 (16), 4588–4602.
Si, H., Verma, S.C., Lampson, M.A., Cai, Q., Robertson, E.S., 2008. Kaposi's sarcoma-associated herpesvirus-encoded LANA can interact with the nuclear mitotic apparatus protein to regulate genome maintenance and segregation. J. Virol. 82 (13), 6734–6746.
Stedman, W., Deng, Z., Lu, F., Lieberman, P.M., 2004. ORC, MCM, and histone hyperacetylation at the Kaposi's sarcoma-associated herpesvirus latent replication origin. J. Virol. 78 (22), 12566–12575.
Sugden, B., 2002. In the beginning: a viral origin exploits the cell. Trends Biochem. Sci. 27 (1), 1–3.
Sun, W.H., Coleman, T.R., DePamphilis, M.L., 2002. Cell cycle-dependent regulation of the association between origin recognition proteins and somatic cell chromatin. EMBO J. 21 (6), 1437–1446.
Takayama, M., Taira, A.T., Tamai, K., Iguchi-Ariga, S.M., Ariga, H., 2000. ORC1 interacts with c-myc to inhibit E-box-dependent transcription by abrogating c-Myc-SNF5/INI1 interaction. Genes Cells 5, 481–490.
Ueda, K., Sakakibara, S., Ohsaki, E., Yada, K., 2006. Lack of a mechanism for faithful partition and maintenance of the KSHV genome. Virus Res. 122 (1–2), 85–94.
Vashee, S., Cvetic, C., Lu, W., Simancek, P., Kelly, T.J., Walter, J.C., 2003. Sequence-independent DNA binding and replication initiation by the human origin recognition complex. Genes Dev. 17 (15), 1894–1908.
Verma, S.C., Choudhuri, T., Kaul, R., Robertson, E.S., 2006. Latency-associated nuclear antigen (LANA) of Kaposi's sarcoma-associated herpesvirus interacts with origin recognition complexes at the LANA binding sequence within the terminal repeats. J. Virol. 80 (5), 2243–2256.
Vogelstein, B., Pardoll, D.M., Coffey, D.S., 1980. Supercoiled loops and eucaryotic DNA replication. Cell 22 (1 Pt 1), 79–85.
Wei, X., Somanathan, S., Samarabandu, J., Berezney, R., 1999. Three-dimensional visualization of transcription sites and their association with splicing factor-rich nuclear speckles. J. Cell Biol. 146 (3), 543–558.

J Gastroenterol 2008; 43:942–950
DOI 10.1007/s00535-008-2256-8

Journal of Gastroenterology

Association of a genetic polymorphism in ectonucleotide pyrophosphatase/phosphodiesterase 1 with hepatitis C virus infection and hepatitis C virus core antigen levels in subjects in a hyperendemic area of Japan

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Background. The clinical course of chronic hepatitis C virus (HCV) infection is strongly associated with insulin resistance and obesity. The K121Q polymorphism in the ectonucleotide pyrophosphatase/phosphodiesterase (*ENPP*)-1 gene and the rs7566605 genotype located near insulin-induced gene 2 have been shown to be associated with insulin resistance and obesity. This study examined whether the K121Q polymorphism in *ENPP1* or the rs7566605 genotype is associated with the clinical course of HCV infection. ***Methods.*** The relationships between the clinical characteristics of 469 anti-HCV antibody-seropositive subjects (353 were positive for HCV core antigen or RNA, whereas 116 were negative for HCV RNA) and the polymorphisms were analyzed. ***Results.*** No significant differences in body mass index, plasma glucose level, serum insulin level, and other biochemical markers were observed between subgroups of subjects with different genotypes at the K121Q polymorphism or rs7566605. The frequency of the homozygous wild-type genotype at K121Q in HCV carriers, however, was significantly higher than that in subjects who were negative for HCV RNA (84.5% vs. 75.9%; $P < 0.05$). Moreover, in HCV carriers, HCV core antigen levels in subjects homozygous for the wild-type genotype at K121Q were significantly higher than in heterozygous carriers of K121Q (5358 fmol/l vs. 4002 fmol/l; $P = 0.04$). In contrast, the rs7566605 genotype was not associated with hepatitis C viremia or with the HCV core antigen level. ***Conclusions.*** The K121Q variant of *ENPP1* may be associated with hepatitis C viremia and core antigen levels in HCV carriers.

Received: March 10, 2008 / Accepted: July 3, 2008
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Key words: hepatitis C virus, *ENPP1*, insulin resistance, viremia, single nucleotide polymorphism, HCV core antigen

Introduction

Hepatitis C virus (HCV) infection, a major cause of chronic hepatitis, may progress to cirrhosis or hepatocellular carcinoma (HCC). Persistent HCV infection can be detected in the sera of 50%–80% of subjects positive for anti-HCV antibodies; in contrast, 20%–50% of those subjects are consistently negative for HCV RNA, suggesting that they have successfully eliminated the HCV infection.[1] Factors such as ethnicity, icteric clinical presentation, absence of human immunodeficiency virus (HIV) infection, and specific HLA type II alleles have been shown to be associated with viral clearance.[2–4] Even in the absence of these factors, however, viral clearance may occur, suggesting the presence of other unidentified cofactors.

Being overweight or obese is an independent risk factor for hepatic steatosis, which accelerates the activity and progression of chronic hepatitis C (CHC).[5] Another risk factor for steatosis is insulin resistance, which is associated with advanced fibrosis and hyporesponsiveness to antiviral therapy.[6] Although obesity and insulin resistance are known to be caused by a combination of genetic and environmental factors, the impact of genetic factors on the clinical course of HCV infection or the severity of liver disease has not been fully elucidated.

A number of reports indicate that single nucleotide polymorphisms (SNPs) in the gene encoding the K121Q variant of ectonucleotide pyrophosphatase/phosphodi-

esterase 1 (*ENPP1*, also known as PC-1) influence insulin resistance, type 2 diabetes, and obesity.[7–11] Recently, the rs7566605 genotype, which is located near the gene encoding insulin-induced gene 2 (*INSIG2*), was also shown to be strongly associated with insulin resistance.[12] Other studies, however, have reported no significant associations between the K121Q variant and insulin resistance or type 2 diabetes,[13–15] and the association between the K121Q variant or rs7566605 genotype and the clinical features of patients with chronic HCV infection has not been fully evaluated.

We examined the natural history of HCV infections in an adult Japanese community-based population in an HCV hyperendemic area beginning in 1994.[16,17] Because movement of the residents in or out of this region is rare, this area provided an appropriate setting to investigate the effects of a genetic background on HCV infections. In this study, we sought to determine the prevalence of the rs7566605 genotype and polymorphisms of the *ENPP1* gene encoding the K121Q variant and to assess their relationship with body mass index (BMI), insulin resistance, and the clinical characteristics of subjects positive for anti-HCV antibodies in an HCV hyperendemic area in Japan.

Materials and methods

Study population

We evaluated 459 anti-HCV antibody-seropositive subjects. Among these subjects, 343 were positive for HCV RNA or HCV core antigen (HCV carrier group), and 116 were negative for both HCV RNA and HCV core antigen (HCV RNA-negative group). All the subjects were Japanese and lived in an HCV hyperendemic area (Town C).[16–18] The Town C HCV study is a cohort study examining the natural course of HCV infections in adult residents of a community in Miyazaki Prefecture, Japan. Residents who were identified as anti-HCV antibody positive at general health examinations were invited to participate in annual examinations for liver disease. No one in this study population had received interferon therapy or was positive for hepatitis B surface antigen. Informed consent was obtained from all participants at the time of enrollment. This study was approved by the human subjects committees of the University of Miyazaki (Faculty of Medicine, Japan), the Harvard School of Public Health, and the Boston University School of Public Health.

Blood tests for hepatic fibrosis markers, anti-HCV antibodies, and HCV core antigen levels

Serum anti-HCV antibodies were detected using chemiluminescence enzyme immunoassays and a third-generation kit (Lumipulse Ortho II; Ortho-Clinical Diagnostics, Tokyo, Japan) at least once for each subject between 2001 and 2003. Additionally, 301 subjects in the HCV carrier group and 100 subjects in the HCV RNA-negative group were known to be positive for anti-HCV antibodies before 1996 as a result of second-generation enzyme immunoassay testing (Immunocheck F-HCV Ab; International Reagents, Kobe, Japan).[16–19] The presence of serum HCV RNA was determined using qualitative reverse transcription-polymerase chain reaction (RT-PCR) (Amplicore HCV; Nippon Roche, Tokyo, Japan). HCV core antigen levels were measured using immunoradiometric assays and a cutoff value for a positive result of 20 fmol/l (Ortho HCV Ag IRMA test; Ortho-Clinical Diagnostic). The levels of plasma glucose (normal range, 70–109 mg/dl), serum insulin (≤17 mU/ml), aspartate aminotransferase (AST) (10–40 IU/l), alanine aminotransferase (ALT) (5–40 IU/l), γ-glutamyl transpeptidase (GTP) (female: 7–30 IU/l; male: 7–70 IU/l), ferritin (female: 7–110 mg/dl; male: 24–286 mg/dl), and the platelet count (12.0–34.0 × 10^4 cells/μl) were examined in each patient. The HCV serotype of each subject was determined before 2001. If the HCV serotype was not determined, the HCV genotype was examined (HCV Core Genotype; SRL, Tokyo, Japan). HCV genotype 1b was considered to be serotype I and genotypes 2a and 2b were considered to be serotype II. No other HCV genotype was detected in this study. Insulin resistance was assessed using a homeostasis model assessment of insulin resistance (HOMA-IR). HOMA-IR values were calculated as follows: plasma glucose (mg/dl) × serum insulin (mU/ml)/405. Hyaluronic acid and type IV collagen 7S, which are known to be hepatic fibrosis markers, were examined using a latex bead agglutination assay (LPIA-ACE HA; Mitsubishi Kagaku Iatron, Tokyo, Japan; normal range: ≤50 ng/ml) and a radioimmunoassay (Type IV collagen 7S kit; Mitsubishi Kagaku Iatron; normal range: ≤6.0 ng/ml), respectively.

DNA extraction and real-time PCR allelic discrimination assays

DNA extraction and real-time PCR allelic discrimination assays were carried out as described previously.[19] Briefly, 10 μl whole blood was drawn into an ethylenediaminetetraacetic acid (EDTA)-containing Vacutainer by venipuncture. Genomic DNA was extracted from the buffy coat fraction, which was separated from the blood by centrifugation at 3000 rpm using Mag-Extractor System MFX-2000 (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The *ENPP1* K121Q SNP was examined using PCR and sequence-specific primers. Real-time PCR allelic discrimination assays were designed using TaqMan SNP genotyping

assays (Applied Biosystems, Foster City, CA, USA). Assays were performed to genotype the A→C SNP corresponding to *ENPP1* K121Q using commercially available primers (dbSNP ID: rs1044498; TaqMan SNP genotyping assays ID: C_1207994_20). We also evaluated the rs7566605 genotype located near the *INSIG2* gene.[12] Genotyping of the G→C SNP (rs7566605) was performed with the primers rs7566605-F (AGTAGGGTGAGGAAACCAAATTCTC) and rs7566605-R (CATGACCCCTACCGTCTCTATTTT), and the probes rs7566605-VIC (ACAGAGATGTTA CATCAC labeled with the dye VIC) and rs7566605-FAM (CACAGAGATATTACATCAC labeled with the dye FAM) in a custom TaqMan genomic assay. Briefly, 5 ng DNA was mixed with TaqMan Universal PCR master mix (Applied Biosystems) and allelic discrimination assay mix (900 nM each primer and 200 nM each FAM or VIC-labeled probe). PCRs were carried out in a total volume of 6 or 10 µl in 96-well PCR plates. The PCR conditions were as follows: 50°C for 2 min for contamination control with AmpErase uracil-*N*-glycosylase and 95°C for 10 min to activate the AmpliTaq Gold enzyme, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. Genotypes were assessed using the TaqMan allele-specific assay method and an ABI Prism 7000 sequence detection system according to the manufacturer's protocol (Applied Biosystems). All genotypes were scored using the allelic discrimination program from the ABI software.

Statistical evaluation

The differences in mean values were assessed using Mann–Whitney *U* tests. Fisher's exact tests and χ^2 tests were used where appropriate. Univariate and multivariate logistic regression analyses were also used to determine the factors that significantly associated with viral clearance or viral load. All statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA, USA) or SPSS version 11.01 statistical analysis software (SPSS, Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

Results

Characteristics of the subjects

The clinical characteristics of the study population are shown in Table 1. In this study, 343 subjects were positive for anti-HCV antibodies and the presence of HCV RNA and/or HCV core antigen (HCV carrier group), whereas 116 subjects were positive for anti-HCV antibodies but were negative for both HCV RNA and HCV core antigen (HCV RNA-negative group). The mean age of the subjects was 70 years (range, 42–97 years old), and the mean BMI of the subjects positive for anti-HCV antibodies was 23 kg/m^2 (range, 15.6–33.5 kg/m^2). Although there were no differences in the distribu-

Table 1. Clinical characteristics of subjects positive for antihepatitis C virus (HCV), according to the presence of hepatitis C viremia

Characteristics	HCV carrier[a] (n = 343)	HCV RNA-negative[b] (n = 116)	P value[c]
Age (years)	70.7 ± 9.7	69.6 ± 11.2	0.67
Sex (male/female)	117/226	37/79	0.66
History of alcohol consumption (daily/occasionally/none)[d]	110/23/174	35/7/63	0.83
Past history of BT (yes/no)[d]	50/273	25/83	0.07
HCV core antigen	4871.6 ± 4869.4 (325)	–	–
HCV serotype (I/II)[e]	225/118	–	–
Body mass index	23.1/1/3.0 (286)	23.1 ± 3.3 (93)	0.73
AST (IU/l)	49.4 ± 32.9	26.4 ± 8.6	<0.001
ALT (IU/l)	44.9 ± 38.2	20 ± 10.1	<0.001
γ-GTP (IU/l)	35.0 ± 52.3 (248)	21.6 ± 26.4 (91)	<0.001
PLT (×10^4)	19.1 ± 6.2 (342)	23.8 ± 5.6	<0.001
Tryglyceride (mg/dl)	110.2 ± 57.2 (248)	123.2 ± 59.4 (93)	0.02
Total cholesterol (mg/dl)	170.3 ± 34.7 (248)	193.1 ± 30.8 (93)	<0.001
HbA1c (%)	5.3 ± 0.7 (248)	5.4 ± 1.0 (91)	0.12
Glucose (mg/dl)	97.3 ± 34.4 (273)	95.6 ± 23.6 (88)	0.86
Insulin (µU/ml)	11.4 ± 11.4 (273)	9.3 ± 13.7 (88)	<0.001

Data are shown as means ± SD (number of subjects examined)
BT, blood transfusion; AST, aspartate aminotransferase; ALT, alanine transferase; GTP, guanosine triphosphatase; PLT, platelet count
[a]Positive for HCV RNA or HCV core antigen
[b]Negative for HCV RNA and HCV core antigen
[c]Data were evaluated by χ^2 test, Fischer's exact test, or Mann–Whitney test, as appropriate
[d]Excluding subjects whose history was not available
[e]Including subjects whose HCV genotype was determined even if serotype was undetermined

Table 2. Prevalence of *ENPP1* K121Q genotype or rs7566605 genotype in subjects with positive for anti-HCV, according to the presence of hepatitis C viremia

	HCV carrier[a]	HCV RNA-negative[b]	*P* value[c]
K121Q genotype	$n = 342$	$n = 116$	
AA	289 (84.5%)	88 (75.9%)	
AC	53 (15.5%)	26 (22.4%)	
CC	0	2 (1.7%)	0.01[d]
rs 7566605 genotype	$n = 341$	$n = 116$	
GG	159 (46.6%)	52 (44.8%)	
GC	141 (41.3%)	52 (44.8%)	
CC	41 (12.0%)	12 (10.3%)	0.75

[a] Positive for HCV RNA or HCV core antigen
[b] Negative for HCV RNA and HCV core antigen
[c] Data were analyzed by χ^2 test
[d] *P* value was 0.048 evaluated by subclasses of AA or AC + CC genotype

Table 3. Prevalence of *ENPP1* K121Q genotypes or rs7566605 genotype in HCV carriers, according to the body mass index (BMI)

	Normal weight (BMI <25)	Overweight (BMI ≥25 and <30)	Obesity (BMI ≥30)	*P* value[a]
K121Q genotype	$n = 216$	$n = 76$	$n = 4$ (%)	
AA	182 (84.3%)	66 (86.8%)	3 (75.0%)	
AC	34 (15.7%)	10 (13.2%)	1 (25.0%)	0.75[b]
CC	0	0	0	
rs 7566605 genotype	$n = 216$	$n = 75$	$n = 4$	
GG	107 (49.5%)	30 (40.0%)	2 (50.0%)	
GC	83 (38.4%)	35 (46.7%)	2 (50.0%)	
CC	26 (12.0%)	10 (13.3%)	0	0.36

[a] Data were evaluated by χ^2 test
[b] Data were analyzed excluding CC genotype

tions of age, sex, history of alcohol consumption, BMI, plasma glucose levels, and HbA1c levels between the groups, AST, ALT, γ-GTP, and insulin levels were significantly higher and triglycerides, total cholesterol, and platelet counts were significantly lower in the HCV carrier group than in the HCV RNA-negative group.

Differential distributions of the ENPP1 *K121Q SNP or rs7566605 genotypes and the clinical characteristics*

We successfully genotyped 458 and 457 subjects for the *ENPP1* K121Q SNP and rs7566605, respectively. The *ENPP1* K121Q SNP was differentially distributed between the HCV carrier group and the HCV RNA-negative groups ($P < 0.01$), whereas the rs7566605 genotype was not (Table 2). In univariate analysis, the *ENPP1* K121Q genotypes AC and CC were significantly more prevalent in the HCV RNA-negative group than in the HCV carrier group [odds ratio (OR), 1.74; 95% confidence interval (CI), 1.04–2.91; $P = 0.04$]. No other factors, including age, sex, BMI, history of alcohol consumption, past history of blood transfusion, and the rs7566605 genotype, were significantly different between the groups (data not shown). In multivariate analysis using four factors (age, sex, *ENPP1* K121Q genotype, and rs7566605 genotype), only the *ENPP1* K121Q genotypes AC and CC were associated with being negative for HCV RNA (OR, 1.78; 95% CI, 1.05–2.99; $P = 0.03$).

Relationships between the ENPP1 *K121Q or rs7566605 genotypes and BMI or insulin resistance*

We examined the relationships between the SNPs and available BMI values in HCV carriers: the subjects were classified as overweight (BMI ≥25 and <30 kg/m^2), obese (BMI ≥30 kg/m^2), or normal (BMI <25 kg/m^2). The distributions of the *ENPP1* K121Q and rs7566605 genotypes were similar in all three BMI subgroups (Table 3). In addition, there was no association between these two SNPs and fasting plasma glucose levels greater than 126 mg/dl or a history of diabetes (data not shown). Then, subjects with fasting plasma glucose levels less than 126 mg/dl were selected, and the relationship between the SNPs and insulin resistance was studied after classifying the subjects as insulin resistant (HOMA-IR value ≥2) or not (HOMA-IR value <2). The distributions of the *ENPP1* K121Q and rs7566605

Table 4. Prevalence of *ENPP1* genotypes or rs7566605 genotypes in HCV carriers, according to insulin resistance

	Lower HOMA-IR index (<2)	High HOMA-IR index (≥2)	*P* value[a]
K121Q genotype	$n = 130$	$n = 106$	
AA	106 (81.5%)	94 (88.7%)	
AC	24 (18.5%)	12 (11.3%)	0.13[c]
CC	0	0	
rs 7566605 genotype	$n = 131$	$n = 105$	
GG	68 (51.9%)	48 (45.7%)	
GC	47 (35.9%)	48 (45.7%)	
CC	16 (12.2%)	9 (8.6%)	0.27

HOMA, homeostasis model assessment of insulin resistance
[a] Data were valuated by χ^2 test
[b] Data were analyzed excluding CC genotype

Table 5. Clinical and virological characteristics in individuals who are HCV carriers, according to the *ENPP1* K121Q genotype

Characteristics	*ENPP1* K121Q genotype[a]		*P* value[b]
	AA ($n = 289$)	AC ($n = 53$)	
Age (years)	70.9 ± 9.5	69.7 ± 10.5	0.43
Sex (male/female)	101/188	15/38	0.35
Body mass index	23.1 ± 3.0 (251)	22.8 ± 3.1 (45)	0.44
Alcohol consumption (daily/occasionally/none)[c]	100/22/157	18/4/30	0.98
Past history of blood transfusion (yes/no)[c]	39/234	11/38	0.15
HCV core antigen (fmol/l)[d]	5358.3 ± 4906.7 (272)	4001.8 ± 4526.4 (53)	0.04
HCV core antigen (<1000/≥1000)[e]	73/216	18/35	0.19
HCV serotype (I/II)[f]	182/107	42/11	0.02
AST (IU/l)	49.9 ± 34.4	46.7 ± 23.4	0.83
ALT (IU/l)	45.9 ± 40.5	40.2 ± 21.7	0.86
γ-GTP (IU/l)	36.2 ± 55.0 (210)	28.1 ± 32.5 (38)	0.75
PLT ($\times 10^4$)	19 ± 6.1 (288)	20.0 ± 6.7	0.30
TG (mg/dl)	110.1 ± 57.1 (210)	110.6 ± 58.6 (38)	0.92
Total cholesterol (mg/dl)	170.0 ± 35.0 (210)	172.3 ± 33.2 (38)	0.66
HbA1c (%)	5.3 ± 0.7 (210)	5.4 ± 0.9 (38)	0.67
Glucose (mg/dl)	98.0 ± 35.4 (230)	93.7 ± 28.9 (42)	0.20
Insulin (μU/ml)	11.6 ± 11.7 (230)	10.9 ± 10.2 (42)	0.59
Ferritin (mg/dl)	151.0 ± 215.5	138.5 ± 182.3	0.33
HA (ng/ml)	196.9 ± 365.9 (287)	236.4 ± 391.8	0.58
Type IV collagen 7S (ng/ml)	5.0 ± 1.8 (287)	5.0 ± 2.0	0.39

Data are shown as means ± SD (number of subjects examined)
[a] There was no subject with CC genotype in persistent HCV infection group
[b] Data were evaluated by χ^2 test, Fischer's exact test, or Mann–Whitney test, as appropriate
[c] Excluding subjects whose history was not available
[d] Excluding subjects whose HCV core antigen level was below the cutoff value
[e] Including subjects whose HCV core antigen level was below the cutoff values
[f] Including subjects whose HCV genotype was determined even if serotype was undetermined

genotypes were also similar in the HOMA-IR subgroups (Table 4).

Clinical and biochemical characteristics of the HCV carriers classified based on the ENPP1 *K121Q or rs7566605 genotype*

In the HCV carrier group, biochemical markers from the subjects with AA and AC genotypes at the *ENPP1* K121Q SNP were compared (Table 5). We did not identify any subjects in the HCV carrier group with a CC genotype at this locus. The levels of HCV core antigen in subjects with an AA genotype were higher than in subjects with an AC genotype. The frequency of serotype II was also higher in subjects with an AA genotype than in subjects with an AC genotype. No other clinical or biochemical characteristics were different between the subjects with the different K121Q genotypes.

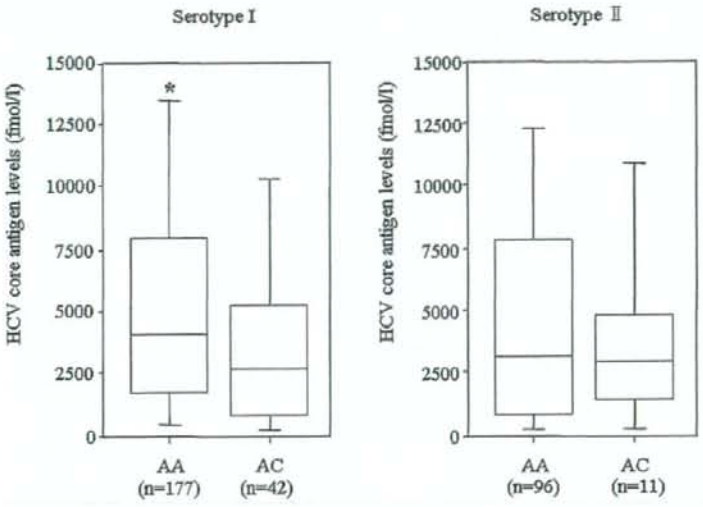

Fig. 1. The association between the K121Q genotype in *ENPP1* and the hepatitis C viral (HCV) load. The box-and-whisker plot shows the HCV core antigen level in the HCV carrier group according to the genotypes. The *boxes* indicate the 25th, 50th (median), and 75th percentiles. The *whiskers* indicate the 10th and 90th percentiles. The *asterisk* refers to a statically significant difference between the HCV core antigen levels in patients with the AA or AC genotype (Mann–Whitney *U* test, $*P = 0.04$)

We then further analyzed the association between the *ENPP1* K121Q variant and HCV core antigen levels according to the HCV serotype (Fig. 1). In the subgroup of subjects classified as HCV serotype I, the hepatitis C viral load was significantly higher in the subjects with the AA genotype (the wild-type genotype) than in those with the AC genotype ($P = 0.04$). Five subjects with the AA genotype were not included in this comparison because their levels of HCV core antigen were below the threshold. In any case, the percentage of subjects with HCV core antigen levels below the cutoff value of 1000 fmol/l was lower in the AA genotype subgroup than in the AC genotype subgroup (23.0% vs. 61.5%, $P < 0.01$ calculated using Fisher's exact test; OR, 2.68; 95% CI, 1.30–5.54; $P < 0.01$). Although a past history of blood transfusion was also associated with HCV core antigen levels (OR, 2.75; 95% CI, 1.25–6.06; $P = 0.01$), no other factors were associated with this variable. In multivariate analysis using the *ENPP1* K121Q variant and past history of blood transfusion, these two factors were independently associated with low HCV core antigen levels (OR, 2.44; 95% CI, 1.12–5.32; $P = 0.03$ and OR, 2.56; 95% CI 1.14–5.72; $P = 0.02$, respectively). This correlation between the HCV core antigen levels and the K121Q genotype, however, was not observed in the subgroup of subjects classified as HCV serotype II (Fig. 1).

In addition, we compared the biochemical markers from the subjects with the GG, GC, and CC genotypes at rs7566605. There were no significant differences among the clinical or biochemical characteristics of the subjects from these three groups, including the viral load (data not shown).

Discussion

Obesity and insulin resistance, which are caused by a combination of genetic and environmental factors, affect the clinical course of CHC infection.[5,6] The K121Q polymorphisms in the *ENPP1* gene and the rs7566605 genotype have been shown to be significantly associated with obesity and insulin resistance.[7–12] Whether polymorphisms in genes associated with obesity or insulin resistance affect persistent HCV infection or HCV-induced liver injury, however, has yet to be determined. We sought to examine the relationship between polymorphisms in these types of genes and viremia or the clinical course of liver injury in subjects positive for anti-HCV antibodies in a community-based HCV hyperendemic area in Japan. Our study, which shows that polymorphisms associated with the K121Q variant and the rs7566605 genotype are prevalent in Japan, suggests that these genotypes are not associated with obesity or insulin resistance in the examined HCV hyperendemic area. In addition, these polymorphisms were not associated with HCV-induced liver injury. In contrast, the frequencies of the K121Q polymorphism in subjects with hepatitis C viremia and those without viremia were different. Moreover, the K121Q polymorphism was associated with HCV viral load in a subgroup of HCV carriers (serotype I).

ENPP1 is the best characterized of the five human ectoenzyme *ENPP* proteins. *ENPP1* is expressed in many tissues, including muscle, fat, and liver, and overexpression of *ENPP1* in various cell lines inhibits insulin receptor tyrosine kinase activity and causes insulin resistance.[20] It was also reported that the K121Q variant

of *ENPP1* is associated with insulin resistance.[21,22] Compared to the *ENPP1* K121 protein, the *ENPP1* Q121 variant interacts more strongly with the insulin receptor and more effectively inhibits insulin-stimulated insulin receptor autophosphorylation and insulin receptor substrate-1 phosphorylation in vitro.[23] In our study, however, there was no association between the *ENPP1* K121Q variant and insulin resistance in HCV carriers. Keshavarz et al. also failed to find evidence of an association between the *ENPP1* K121Q variant and type 2 diabetes in a Japanese population.[24] The overall frequency of the 121Q allele (9.1%; 83/916) in our study was similar to that in the Japanese population, as previously reported (10.5%; 375/3562).[24] These results indicate that our study population represented the rest of Japan and that the K121Q variant does not influence insulin resistance in Japanese subjects, in particular in subjects with HCV infections.

rs7566605 is upstream of the transcription start site of *INSIG2*, the protein product of which inhibits the synthesis of fatty acids and cholesterol.[25] Overexpression of *INSIG2* in the liver reduced plasma triglyceride levels in obese Zucker diabetic fatty rats, and linkage between this gene and obesity phenotypes was observed in the mice.[26,27] Association testing in nine cohorts produced evidence that individuals with the CC genotype at rs7566605 have higher BMI values and a higher risk of obesity than those with the GG or GC genotype.[28] More recently, however, no association was reported between this genotype and obesity.[29,30] In addition, the rs7566605 genotype was not associated with the clinical or biochemical characteristics of subjects positive for anti-HCV antibodies, obesity, or insulin resistance in our study. These conflicting results about the relationship between the rs7566605 genotype and BMI may have resulted from the heterogeneous population samples. Future studies should enroll a large number of patients with HCV infections and control subjects from throughout the Japanese population.

False-positive results for the HCV antibody test may have occurred in the HCV RNA-negative group in our study. Several studies have shown that samples with readings just slightly above the cutoff value of the anti-HCV test have a greater likelihood to be false-positives compared with those with higher values.[31,32] HCV-positive patients may also show reactivity to nuclear and smooth muscle antigens.[33,34] There was, however, no difference in the distributions of the *ENPP1* K121Q genotypes (AA, AC, or CC) among patients with low titers (≥1 and <5), intermediate titers (≥5 and <30), and high titers (≥30) of anti-HCV antibodies in our study (data not shown). In addition, although there was no evidence of spontaneous clearance of HCV infection in this study, Micallef et al. systematically reviewed 31 longitudinal studies with a total of 675 subjects and reported that spontaneous viral clearance occurs in approximately one in four people with acute hepatitis C, which was similar to the size of the HCV RNA-negative group (25%).[35] Although autoantibody data and evidence of spontaneous HCV clearance in the clinical courses are not available, these results indicate that many subjects in the HCV RNA-negative group in our study population may have cleared their HCV infection spontaneously without false-positive results for the HCV antibody test.

Spontaneous HCV clearance typically occurs within the first 6 months after acute infection,[36] and spontaneous elimination of HCV in subjects with chronic HCV infection is rare.[16] These results suggest that *ENPP1* may influence the spontaneous clearance of HCV during the acute phase of infection in our population. Furthermore, sex is known to be an important factor for HCV clearance,[37–39] although a sex-based difference was not observed in our study (see Table 1). Studies based on polymorphisms have been widely used to identify host genetic factors that influence disease occurrence, progression, and outcome.[40] However, it is unclear whether *ENPP1* and sex are associated in HCV clearance. Another potential confounding variable is alcohol use, which is known to be negatively associated with HCV clearance.[41] Alcohol use, however, is limited in this community, and thus was unlikely to be a confounder. Further studies are needed to clarify the associations between host factors and *ENPP1* and their roles in HCV clearance.

Analysis of the *ENPP1* gene in 6147 subjects showed an association between a three-allele risk haplotype (K121Q, IVS20delT-11, and A→G+1044TGA) and obesity and type 2 diabetes.[42] In that report, it was shown that the presence of at least one copy each of the Gln121(121Q), IVS20delT-11, and G+1044TGA variants was associated with a significant increase in serum *ENPP1* protein levels. In addition, serum levels of osteopontin were lower in *ENPP1*-deficient mice than in wild-type mice, suggesting that *ENPP1* affects osteopontin expression.[43] Osteopontin-deficient mice also suffered from prolonged rotavirus-induced diarrhea.[44] SNPs in the promoter region of the osteopontin gene have been identified as markers that predict the efficacy of interferon-based therapies in patients with CHC.[45] Although our studies do not directly identify increased serum levels of *ENPP1* or osteopontin, *ENPP1* may induce nonproductive binding of HCV to cells, blockade of HCV attachment, or inhibition of penetration into cells through osteopontin expression.

The precise roles that host factors play in HCV replication have not been well characterized. Although Woitas et al. reported that anti-HCV-antibody-seropositive patients who were homozygous for the HIV-protective CC chemokine receptor (CCR) 5-Δ32

showed a markedly increased viral load compared with CCR5 wild-type or CCR5-Δ32 heterozygous patients,[46] the authors did not show results based on the HCV genotype or serotype. Hepatitis C viral load was found to be significantly higher in patients infected with HCV genotype 1 compared to patients infected with HCV genotype 2 or 3.[47] Our study indicates that the AC genotype at the K121Q SNP of *ENPP1* is linked to lower HCV core antigen levels, which correlated with hepatitis C viral load in the HCV serotype I subgroup, but not in the serotype II subgroup. The mechanisms contributing to the relationship between the K121Q polymorphism and the hepatitis C viral load are unclear. HCV replication in the cytoplasm, however, is highly dependent on the functions of nonstructural HCV proteins together with those of host factors.[48,49] Thus, functional studies about the molecular mechanisms underlying *ENPP1* signaling in HCV replication should be conducted in the future.

Acknowledgments. This work was supported by a grant (No. CA87982) from the United States National Institutes of Health; a grant-in-aid (Research on Hepatitis and BSE) from the Ministry of Health, Labour and Welfare of Japan; and a grant from the Miyazaki Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence (Japan Science and Technology Corporation). We thank Ms. Keiko Toyama and Ms. Yuko Nakamura for their technical assistance.

References

1. Seeff LB. Natural history of chronic hepatitis C. Hepatology 2002;36:S35–46.
2. Villano SA, Vlahov D, Nelson KE, Cohn S, Thomas DL. Persistence of viremia and the importance of long-term follow-up after acute hepatitis C infection. Hepatology 1999;29:908–14.
3. Thomas DL, Astemborski J, Rai RM, Anania FA, Schaeffer M, Galai N, et al. The natural history of hepatitis C virus infection: host, viral, and environmental factors. JAMA 2000;284:450–56.
4. Thursz M, Yallop R, Goldin R, Trepo C, Thomas HC. Influence of MHC class II genotype on outcome of infection with hepatitis C virus. Lancet 1999;354:2119–24.
5. Kenny-Walsh E for the Irish Hepatology Research Group. Overweight and obesity, hepatic steatosis, and progression of chronic hepatitis C: a retrospective study on a large cohort of patients in the United States. J Hepatol 2004;40:147–54.
6. Cammà C, Bruno S, Di Marco V, Di Bona D, Rumi M, Vinci M, et al. Insulin resistance is associated with steatosis in nondiabetic patients with genotype 1 chronic hepatitis C. Hepatology 2006;43: 64–71.
7. Abate N, Chandalia M, Satija P, Adams-Huet B, Grundy SM, Sandeep S, et al. ENPP1/PC-1 K121Q polymorphism and genetic susceptibility to type 2 diabetes. Diabetes 2005;54:1207–13.
8. Bacci S, Ludovico O, Prudente S, Zhang YY, Di Paola R, Mangiacotti D, et al. The K121Q polymorphism of the ENPP1/PC-1 gene is associated with insulin resistance/atherogenic phenotypes, including earlier onset of type 2 diabetes and myocardial infarction. Diabetes 2005;54:3021–25.
9. Grarup N, Urhammer SA, Ek J, Albrechtsen A, Glumer C, Borch-Johnsen K, et al. Studies of the relationship between the ENPP1 K121Q polymorphism and type 2 diabetes, insulin resistance and obesity in 7,333 Danish white subjects. Diabetologia 2006;49:2097–104.
10. Böttcher Y, Körner A, Reinehr T, Enigk B, Kiess W, Stumvoll M, Kovacs P. ENPP1 variants and haplotypes predispose to early onset obesity and impaired glucose and insulin metabolism in German obese children. J Clin Endocrinol Metab 2006;91: 4948–52.
11. Meyre D, Bouatia-Naji N, Tounian A, Samson C, Lecoeur C, Vatin V, et al. Variants of ENPP1 are associated with childhood and adult obesity and increase the risk of glucose intolerance and type 2 diabetes. Nat Genet 2005;37:863–7.
12. Herbert A, Gerry NP, McQueen MB, Heid IM, Pfeufer A, Illig T, et al. A common genetic variant is associated with adult and childhood obesity. Science 2006;312:279–83.
13. Keshavarz P, Inoue H, Sakamoto Y, Kunika K, Tanahashi T, Nakamura N, et al. No evidence for association of the ENPP1 (PC-1) K121Q variant with risk of type 2 diabetes in a Japanese population. J Hum Genet 2006;51:559–66.
14. Weedon MN, Shields B, Hitman G, Walker M, McCarthy MI, Hattersley AT, Frayling TM. No evidence of association of ENPP1 variants with type 2 diabetes or obesity in a study of 8,089 U.K. Caucasians. Diabetes 2006;55:3175–9.
15. Lyon HN, Florez JC, Bersaglieri T, Saxena R, Winckler W, Almgren P, et al. Common variants in the *ENPP1* gene are not reproducibly associated with diabetes or obesity. Diabetes 2006; 55:3180–4.
16. Uto H, Hayashi K, Kusumoto K, Hasuike S, Nagata K, Kodama M, et al. Spontaneous elimination of hepatitis C virus RNA in individuals with persistent infection in a hyperendemic area of Japan. Hepatol Res 2006;34:28–34.
17. Hayashi K, Hasuike S, Kusumoto K, Ido A, Uto H, Kenji N, et al. Usefulness of a new immuno-radiometric assay to detect hepatitis C core antigen in a community-based population. J Viral Hepat 2005;12:106–10.
18. Suruki R, Hayashi K, Kusumoto K, Uto H, Ido A, Tsubouchi H, Stuver SO. Alanine aminotransferase level as a predictor of hepatitis C virus-associated hepatocellular carcinoma incidence in a community-based population in Japan. Int J Cancer 2006;119: 192–5.
19. Kusumoto K, Uto H, Hayashi K, Takahama Y, Nakao H, Suruki R, et al. Interleukin-10 or tumor necrosis factor-alpha polymorphisms and the natural course of hepatitis C virus infection in a hyperendemic area of Japan. Cytokine 2006;34:24–31.
20. Dong H, Maddux BA, Altomonte J, Meseck M, Accili D, Terkeltaub R, et al. Increased hepatic levels of the insulin receptor inhibitor, PC-1/NPP-1, induce insulin resistance and glucose intolerance. Diabetes 2005;54:367–72.
21. Hamaguchi K, Terao H, Kusuda Y, Yamashita T, Hazoury Bahles JA, Cruz LL M, et al. The PC-1 Q121 allele is exceptionally prevalent in the Dominican Republic and is associated with type 2 diabetes. J Clin Endocrinol Metab 2004;89:1359–64.
22. Pizzuti A, Frittitta L, Argiolas A, Baratta R, Goldfine ID, Bozzali M, et al. A polymorphism (K121Q) of the human glycoprotein PC-1 gene coding region is strongly associated with insulin resistance. Diabetes 1999;48:1881–4.
23. Costanzo BV, Trischitta V, Di Paola R, Spampinato D, Pizzuti A, Vigneri R, Frittitta L. The Q allele variant (GLN121) of membrane glycoprotein PC-1 interacts with the insulin receptor and inhibits insulin signaling more effectively than the common K allele variant (LYS121). Diabetes 2001;50:831–6.
24. Keshavarz P, Inoue H, Sakamoto Y, Kunika K, Tanahashi T, Nakamura N, et al. No evidence for association of the ENPP1 (PC-1) K121Q variant with risk of type 2 diabetes in a Japanese population. J Hum Genet 2006;51:559–66.
25. Yabe D, Brown MS, Goldstein JL. Insig-2, a second endoplasmic reticulum protein that binds SCAP and blocks export of sterol regulatory element-binding proteins. Proc Natl Acad Sci USA 2002;99:12753–8.

26. Takaishi K, Duplomb L, Wang MY, Li J, Unger RH. Hepatic insig-1 or -2 overexpression reduces lipogenesis in obese Zucker diabetic fatty rats and in fasted/refed normal rats. Proc Natl Acad Sci U S A 2004;101:7106–11.
27. Deng HW, Deng H, Liu YJ, Liu YZ, Xu FH, Shen H, et al. A genomewide linkage scan for quantitative-trait loci for obesity phenotypes. Am J Hum Genet 2002;70:1138–51.
28. Lyon HN, Emilsson V, Hinney A, Heid IM, Lasky-Su J, Zhu X, et al. The association of a SNP upstream of *INSIG2* with body mass index is reproduced in several but not all cohorts. PLoS Genet 2007;27:627–33.
29. Kumar J, Sunkishala RR, Karthikeyan G, Sengupta S. The common genetic variant upstream of INSIG2 gene is not associated with obesity in Indian population. Clin Genet 2007;71: 415–8.
30. Smith AJ, Cooper JA, Li LK, Humphries SE. INSIG2 gene polymorphism is not associated with obesity in Caucasian, Afro-Caribbean and Indian subjects. Int J Obes (Lond) 2007;31: 1753–5.
31. Goncales NS, Costa FF, VAssallo J, Concales FL. Diagnosis of hepatitis C virus in Brazilian blood donors using a reverse transcriptase nested polymerase chain reaction: comparison with enzyme immunoassay and recombinant protein immunoblot assay. Rev Inst Med Trop Sao Paulo 2000;42:263–7.
32. Schröter M, Schäfer P, Zöllner B, Polywka S, Laufs R, Feucht HH. Strategies for reliable diagnosis of hepatitis C infection: the need for a serological confirmatory assay. J Med Virol 2001;64: 320–4.
33. Nishiguchi S, Kuroki T, Ueda T, Fukuda K, Takeda T, Nakajima S, et al. Detection of hepatitis C virus antibody in the absence of viral RNA in patients with autoimmune hepatitis. Ann Intern Med 1992;116:21–5.
34. Gregorio GV, Choudhuri K, Ma Y, Pensati P, Iorio R, Grant P, et al. Mimicry between the hepatitis C virus polyprotein and antigenic targets of nuclear and smooth muscle antibodies in chronic hepatitis C virus infection. Clin Exp Immunol 2003;133: 404–13.
35. Micallef JM, Kaldor JM, Dore GJ. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. J Viral Hepat 2006;13:34–41.
36. Hoofnagle JH. Course and outcome of hepatitis C. Hepatology 2002;36:S21–9.
37. Inoue G, Horiike N, Michitaka K, Onji M. Hepatitis C virus clearance is prominent in women in an endemic area. J Gastroenterol Hepatol 2000;15:1054–8.
38. Alric L, Fort M, Izopet J, Vinel JP, Bureau C, Sandre K, et al. Study of host- and virus-related factors associated with spontaneous hepatitis C virus clearance. Tissue Antigens 2000;56:154–8.
39. Bakr I, Rekacewicz C, El Hosseiny M, Ismail S, El Daly M, El-Kafrawy S, et al. Higher clearance of hepatitis C virus infection in females compared with males. Gut 2006;55:1183–7.
40. Hill AV. Immunogenetics and genomics. Lancet 2001;357: 2037–41.
41. Piasecki BA, Lewis JD, Reddy KR, Bellamy SL, Porter SB, Weinrieb RM, et al. Influence of alcohol use, race, and viral coinfections on spontaneous HCV clearance in a US veterans population. Hepatology 2004;40:892–9.
42. Meyre D, Bouatia-Naji N, Tounian A, Samson C, Lecoeur C, Vatin V, et al. Variants of ENPP1 are associated with childhood and adult obesity and increase the risk of glucose intolerance and type 2 diabetes. Nat Genet 2005;37:863–7.
43. Harmey D, Hessle L, Nabisawa S, Johnsof KA, Terkeltaub R, Millán JL. Concerted regulation of inorganic pyrophosphate and osteopontin by akp2, enpp1, and ank: an integrated model of the pathogenesis of mineralization disorders. Am J Pathol 2004; 164:1199–209.
44. Rollo EE, Hempson SJ, Bansal A, Tsao E, Habib I, Rittling SR, et al. The cytokine osteopontin modulates the severity of rotavirus diarrhea. J Virol 2005;79:3509–16.
45. Naito M, Matsui A, Inao M, Nagoshi S, Nagano M, Ito N, et al. SNPs in the promoter region of the osteopontin gene as a marker predicting the efficacy of interferon-based therapies in patients with chronic hepatitis C. J Gastroenterol 2005;40:381–8.
46. Woitas RP, Ahlenstiel G, Iwan A, Rockstroh JK, Brackmann HH, Kupfer B, et al. Frequency of the HIV-protective CC chemokine receptor 5-Δ32/Δ32 genotype is increased in hepatitis C. Gastroenterology 2002;122:1721–8.
47. Blatt LM, Mutchnick MG, Tong MJ, Klion FM, Lebovics E, Freilich B, et al. Assessment of hepatitis C virus RNA and genotype from 6807 patients with chronic hepatitis C in the United States. J Viral Hepatol 2000;7:196–202.
48. Kato N, Lan KH, Ono-Nita SK, Shiratori Y, Omata M. Hepatitis C virus nonstructural region 5A protein is a potent transcriptional activator. J Virol 1997;71:8856–9.
49. Jin DY, Wang HL, Zhou Y, Chun AC, Kibler KV, Hou YD, et al. Hepatitis C virus core protein-induced loss of LZIP function correlates with cellular transformation. EMBO J 2000;19:729–40.