TABLE I. Clinical and Virological Features in 30 Patients With Type B Chronic Hepatitis Treated With Adefovir Dipivoxil Added to Continuing Lamivudine

Patient no.	Age (years)	Gender (M/F)	Liver disease	Duration of preceding lamivudine therapy (months)	ALT (Iμ/I)	HBeAg/ anti-HBe	HBV DNA (log_{10} copies μl)	rtM204V/I mutation (V/I)	rtL180M mutation	V1753 mutation	C2189 mutation	Follow-up period of lamivudine plus adefovir dipivoxil therapy (months)	Sustained HBV DNA clearance	Genbank accession no.
1	51	M	Chronic hepatitis	33	64	+/−	7.2	I	+	−	+	40	+	AB367415
2	54	M	Chronic hepatitis	20	87	+/−	>7.6	V	+	−	+	33	+	AB367393
3	38	M	Chronic hepatitis	30	429	+/−	>7.6	I	+	+ (C)	+	32	+	AB367414
4	33	M	Chronic hepatitis	33	331	+/−	7.5	I	+	+ (C)	+	31	+	AB367413
5	71	F	Cirrhosis	21	148	−/+	7.1	I	−	−	+	31	+	AB367430
6	51	M	Cirrhosis	38	272	−/+	6.9	I	−	+ (G)	+	29	+	AB367804
7	51	M	Chronic hepatitis	36	327	−/+	5.5	V	+	+ (C)	−	28	+	AB367406
8	25	M	Chronic hepatitis	41	455	+/−	6.6	V	+	−	−	27	+	AB367394
9	55	M	HCC	35	96	+/+	5.2	V	+	−	+	26	+	AB367401
10	62	M	Chronic hepatitis	49	401	+/+	7.4	I	−	−	−	15	+	AB367428
11	38	M	Chronic hepatitis	41	122	+/+	7.1	V	+	+ (G)	−	14	+	AB367407
12	27	M	Chronic hepatitis	21	28	+/−	4.9	I	−	+ (A)	−	11	+	AB367422
13	37	F	Chronic hepatitis	54	16	−/+	5.3	I	+	+ (C)	+	6	+	AB367803
14	47	M	Chronic hepatitis	47	59	+/−	>7.6	I	+	−	−	32	−	AB367408
15	36	M	Chronic hepatitis	28	340	+/−	7.1	I	−	−	−	28	−	AB367425
16	39	M	HCC	28	47	+/−	5.0	I	−	−	+	25	−	AB367802
17	64	M	Cirrhosis	45	32	+/−	>7.6	V	+	+ (C)	−	20	−	AB367403
18	60	F	Chronic hepatitis	25	41	−/+	6.6	I	−	−	−	19	−	AB367427
19	57	F	Chronic hepatitis	55	140	+/−	>7.6	I	+	−	−	19	−	AB367410
20	38	M	Chronic hepatitis	28	112	+/−	7.5	V	+	−	+	18	−	AB367398
21	39	M	Chronic hepatitis	35	193	+/−	>7.6	I	+	−	−	17	−	AB367418
22	36	M	Chronic hepatitis	46	115	+/−	7.5	V	+	−	−	17	−	AB367404
23	56	M	HCC	26	35	+/−	>7.6	I	−	−	−	17	−	AB367435
24	46	M	Chronic hepatitis	48	78	−/+	>7.6	V	+	+ (G)	−	16	−	AB367800
25	54	M	Chronic hepatitis	40	104	−/+	7.2	V	+	−	−	13	−	AB367405
26	71	M	Chronic hepatitis	56	184	+/−	7.1	I	−	−	−	11	−	AB367434
27	53	M	HCC	27	120	−/+	6.7	I	−	−	+	10	−	AB367433
28	40	M	Chronic hepatitis	27	56	+/−	7.4	I	−	+ (C)	+	6	−	AB367423
29	63	M	Chronic hepatitis	26	20	+/−	>7.6	V	+	+ (C)	−	6	−	AB367399
30	36	M	Chronic hepatitis	60	66	−/+	6.8	I	−	−	+	6	−	AB367801

50 mM Tris–Cl (pH 7.5), 1 mM EDTA and 1% NP40, followed by the 15-min incubation on ice. Then, the sample was centrifuged to remove the nuclei pellet at 15,000 rpm at 4°C, and treated with 30 μg of DNase I at 37°C for 30 min. The sample was subjected to overnight incubation at 37°C in lysis buffer containing 1% SDS and 200 μg of proteinase K. After phenol/chloroform extraction and ethanol precipitation, the DNA sample was electrophoresed, transferred onto a nylon membrane and hybridized with an alkaline phosphatase-labeled HBV DNA probe. The signals were detected with the chemiluminescent substrate CDP-star (GE Healthcare Bio-Sciences Co. Ltd, Tokyo, Japan) and quantitated using an image analyzing software (ImageJ 1.38, supplied online by the National Institutes of Health, Bethesda, MD).

Statistical Analysis

Group comparisons of continuous and categorical variables were done using Fisher's exact probability test, χ^2 test with Yate's correction and Mann–Whitney's non-parametric *U*-test as appropriate. The group comparison of Kaplan–Meier curves for the cumulative probability of sustained HBV DNA clearance was performed by the log-rank test. The correlation of various clinical and virological factors with the cumulative probability of sustained HBV DNA clearance was evaluated by a Cox proportional-hazards model using univariate and stepwise multivariate procedures. The one-way analysis of variance and the Fisher's PLSD test were used for the in vitro transfection analysis.

RESULTS

Overall Therapeutic Efficacy of Adefovir Dipivoxil Added to Lamivudine in Lamivudine-Resistant Patients With Type B Chronic Hepatitis

Among the lamivudine-resistant patients with type B chronic hepatitis examined in this study, HBV DNA decreased to an undetectable level (<2.6 $\log_{10}$ copies/ml) in 6 (20%) of 30 patients at 6 months, 6 (26%) of 23 patients at 12 months and 9 (56%) of 16 patients at 18 months after the beginning of adefovir dipivoxil administration. Thirteen (43%) of the 30 patients achieved sustained HBV DNA clearance during follow-up. ALT normalization was observed in 21 (70%) of 30 patients at 6 months, 14 (61%) of 23 patients at 12 months and 11 (69%) of 16 patients at 18 months of therapy.

Viral Mutations Associated With Efficacy of Adefovir Dipivoxil Added to Lamivudine Treatment

The lengths of the 30 HBV DNA sequences obtained from the lamivudine-resistant patients with type B chronic hepatitis ranged from 3,161 to 3,230 nucleotides. All 30 patients were infected with HBV of genotype C as determined by phylogenetic tree analysis of the HBV isolates obtained in this study and the representative HBV isolates of major genotypes (data not shown). Viral mutations were sought that showed a relationship with the therapeutic efficacy of adefovir dipivoxil added to lamivudine over the whole HBV genome. As a result, there were only two mutations; one was a T-to-C/G/A mutation at nt1753 (V1753 mutation) located in the basic core promoter (BCP) [Yuh et al., 1992], and the other was an A-to-C mutation at nt2189 (C2189 mutation) in the core gene.

The V1753 mutation was detected in 11 (37%) of the 30 patients studied; the C1753 mutation was found in 7 patients, G1753 in three patients, and A1753 in 1 patient. Figure 1A shows the serial change in HBV DNA before and after the commencement of adefovir dipivoxil administration added to lamivudine treatment in patients with type B chronic hepatitis with and without the V1753 mutation. In patients with V1753, HBV DNA clearance was observed in 5 (45%) of 11 patients at 6 months, 5 (71%) of 7 patients at 12 months and 4 (80%) of 5 patients at 18 months of therapy. By contrast, in patients having T1753, HBV DNA clearance was seen in only 1 (5%) of 19 patients at 6 months, 1 (6%) of 16 patients at 12 months and 5 (45%) of 11 patients at 18 months. Significant differences in the frequency of HBV DNA clearance were observed at 6 and 12 months of therapy between patients with and without V1753 ($P < 0.02$ and $P < 0.005$). Thus, patients with V1753 tended to achieve HBV DNA clearance more frequently by adefovir dipivoxil added to lamivudine treatment than those with T1753.

The C2189 mutation was found in 13 (43%) of the 30 lamivudine-resistant patients with type B chronic hepatitis. Figure 1B shows the serial change in HBV DNA during adefovir dipivoxil administration added to lamivudine treatment in patients with type B chronic hepatitis with and without the C2189 mutation. In patients with C2189, HBV DNA was cleared in 5 (38%) of 13 patients at 6 months, 4 (44%) of 9 patients at 12 months and 8 (89%) of 9 patients at 18 months of therapy. On the other hand, in patients having A2189, HBV DNA was undetectable in 1 (6%) of 17 patients at 6 months, 2 (14%) of 14 patients at 12 months and 1 (14%) of 7 patients at 18 months of therapy. There was a significant difference in the frequency of HBV DNA clearance after 18 months of therapy between patients with and without C2189 ($P < 0.01$). HBV DNA tended to be cleared more frequently by adefovir dipivoxil administration added to lamivudine treatment in patients with C2189 than in those with A2189.

A group comparison of the Kaplan–Meier curves was undertaken for the cumulative probability of sustained HBV DNA clearance with respect to the occurrence of these two viral mutations. Patients with V1753 had a significantly higher cumulative probability of sustained HBV DNA clearance than those with T1753 ($P < 0.005$) (Fig. 2A). The cumulative probability of sustained HBV DNA clearance was also higher in patients with C2189 than in those with A2189 ($P < 0.05$) (Fig. 2B). The presence of the V1753 and C2189 mutations showed no

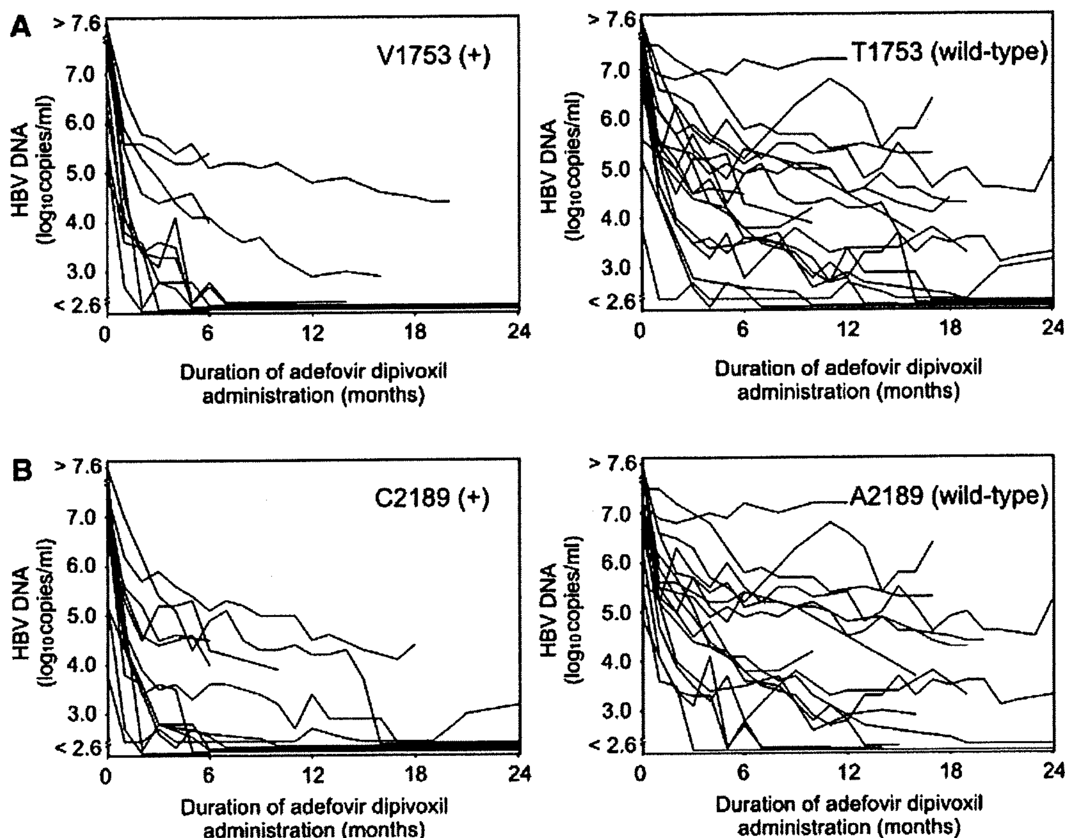

Fig. 1. Serial changes in the HBV DNA level up to 24 months of adefovir dipivoxil added to lamivudine treatment in lamivudine-resistant patients with type B chronic hepatitis in relation to the occurrences of (A) V1753 mutation and (B) C2189 mutation.

relationship with ALT normalization during adefovir dipivoxil added to lamivudine treatment.

Factors Associated With the Efficacy of Adefovir Dipivoxil Added to Lamivudine Treatment Determined by Univariate and Multivariate Analyses

Next, the clinical and virological factors affecting the therapeutic effect of adefovir dipivoxil added to lamivudine were investigated in the 30 patients with type B chronic hepatitis. Six clinical factors (age, gender, liver disease, ALT, HBeAg positivity, and HBV DNA), two lamivudine resistance-associated viral mutations (rtM204V/I and rtL180M) [3], two major naturally occurring viral mutations (A1896 and T1762/A1764) [Carman et al., 1989; Okamoto et al., 1994], and V1753 and C2189 mutations were investigated. As shown in Table II, only the V1753 and C2189 mutations were significant factors contributing to sustained clearance of HBV DNA ($P = 0.006$ and $P = 0.047$) by univariate analysis. High ALT and low HBV DNA (<7.5 $\log_{10}$ copies/ml) at baseline were selected as significant independent factors contributing to sustained clearance of HBV DNA ($P = 0.011$ and $P = 0.008$) in addition to the V1753 and C2189 mutations ($P = 0.001$ and $P = 0.007$) in multivariate analysis.

Serial Changes in the V1753 and C2189 Mutations During Antiviral Therapy

The V1753 and C2189 mutations were also examined using serum samples obtained before lamivudine therapy, which were available in 14 of the 30 type B chronic hepatitis patients. These mutations were assayed by PCR-direct sequencing. Of the 14 patients, the V1753 mutation was found in only 1 patient before lamivudine therapy and detected in additional 4 patients before adefovir dipivoxil administration. The C2189 mutation was found in three patients before lamivudine therapy, one of whom lost the mutation before adefovir dipivoxil administration. The additional three patients acquired this mutation before adefovir dipivoxil administration. Thus, both the V1753 and C2189 mutations tended to appear during lamivudine therapy accompanied by the lamivudine resistance in these patients.

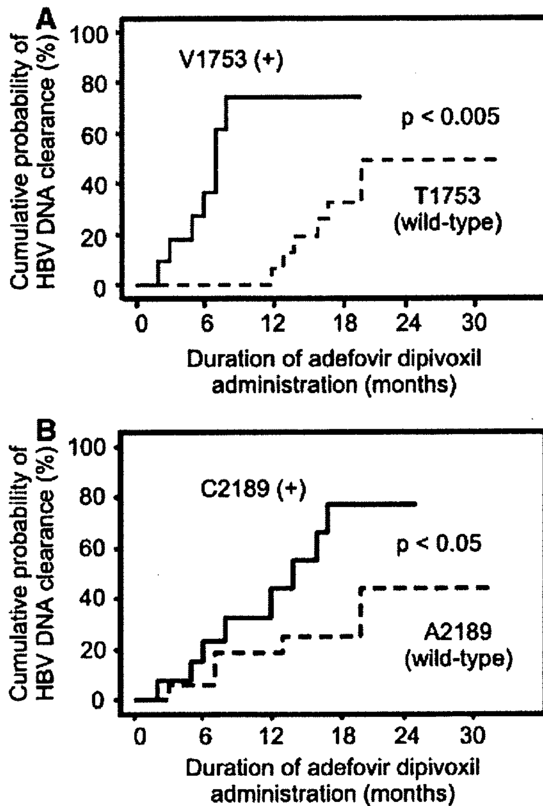

Fig. 2. Influence of the presence of the V1753 and C2189 mutant viruses on the efficacy of adefovir dipivoxil therapy added to lamivudine treatment in lamivudine-resistant patients with type B chronic hepatitis. The Kaplan–Meier estimates of the cumulative probability of sustained HBV DNA clearance correlated with the presence or absence of (A) V1753 mutation and (B) C2189 mutation.

Susceptibility to Lamivudine and/or Adefovir of Wild-Type and Mutant Viruses In Vitro

Finally, susceptibility to lamivudine and/or adefovir of the wild-type and C1753 and C2189 mutant viruses was examined in vitro. Cultured cells with forced expression of the wild-type or mutant virus were treated with lamivudine alone, adefovir alone, lamivudine plus adefovir, or left untreated, and the cellular HBV DNA replicative intermediate was examined. In the HBV-expressing cells without treatment (Fig. 3A,B), the replicative competence of the C1753 mutant virus was the same as that of the wild-type virus, whereas the C2189 mutant virus showed an approximately fivefold lower replicative competence than the wild-type virus. As for susceptibility to nucleos(t)ide analogs, the degree of reduction in viral replication by treatment with lamivudine alone, adefovir alone or lamivudine plus adefovir did not differ significantly among the wild-type virus and the C1753 and C2189 mutant viruses (Fig. 3A,C).

DISCUSSION

Adefovir dipivoxil added to ongoing lamivudine treatment has been accepted as a reliable therapeutic

TABLE II. Univariate and Multivariate Analyses to Investigate Factors Associated With Sustained HBV DNA Clearance in Patients With Type B Chronic Hepatitis Treated With Adefovir Dipivoxil Added to Continuing Lamivudine

	Univariate analysis				Multivariate analysis			
Factors	Hazard ratio	95% confidence interval	χ^2-value	*P*-value	Hazard ratio	95% confidence interval	χ^2-value	*P*-value
Clinical factors								
Age (/1 year increment)	0.987	0.843–1.033	0.302	0.583	—	—	—	—
Gender (female)	1.315	0.287–6.020	0.124	0.725	—	—	—	—
Liver disease (cirrhosis and HCC)	1.199	0.328–4.384	0.076	0.783	—	—	—	—
ALT (/1 IU/l increment)	1.003	1.000–1.007	3.405	0.065	1.005	1.001–1.010	6.443	0.011
HBeAg (negative)	1.919	0.566–6.503	1.096	0.295	—	—	—	—
HBV DNA (<7.5 $\log_{10}$ copies/ml)	3.175	0.862–11.765	3.017	0.082	14.706	1.996–111.111	6.956	0.008
Virological factors								
rtM204V/I mutation (rtM204I)	1.060	0.346–3.254	0.011	0.918	—	—	—	—
rtL180M mutation (+)	0.766	0.234–2.505	0.195	0.659	—	—	—	—
A1896 mutation (+)	1.138	0.371–3.493	0.051	0.821	—	—	—	—
T1762/A1764 mutation (+)	0.500	0.062–4.013	0.426	0.514	—	—	—	—
V1753 mutation (+)	4.986	1.603–15.506	7.705	0.006	58.322	5.054–673.002	10.619	0.001
C2189 mutation (+)	3.155	1.017–9.804	3.957	0.047	7.042	1.704–29.412	7.270	0.007

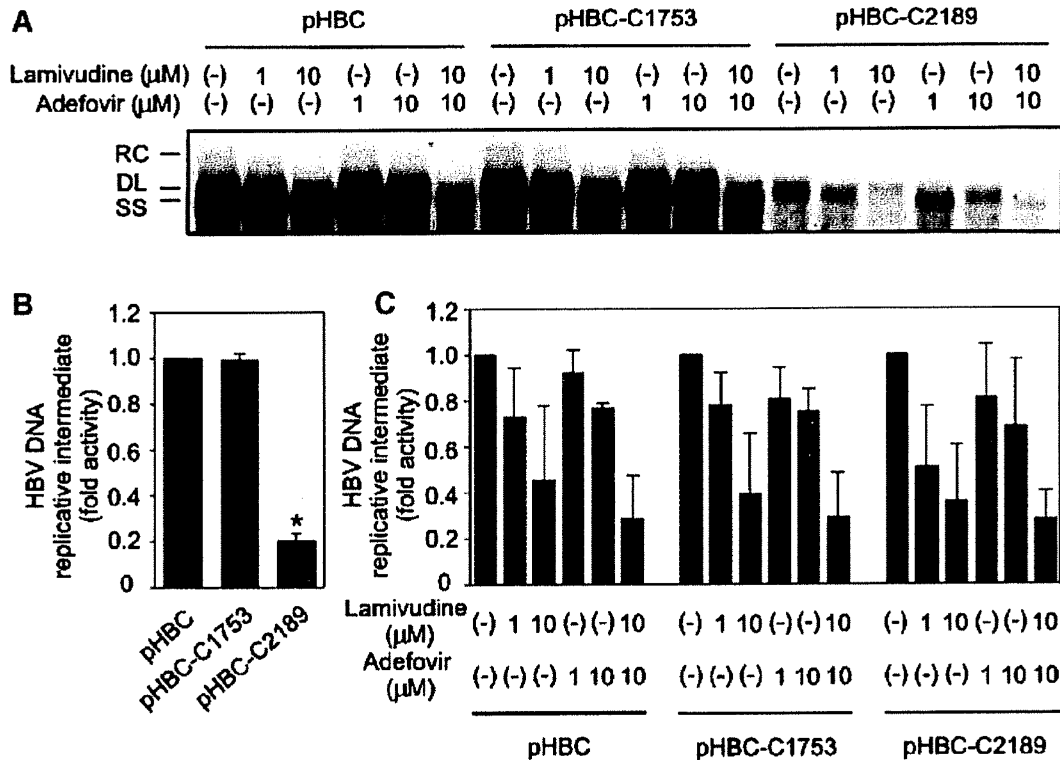

Fig. 3. In vitro transfection analysis to examine viral replicative competence and susceptibility to the treatment with lamivudine and/or adefovir. Huh-7 cells were transfected with pHBC, pHBC-C1753 and pHBC-C2189, and treated with lamivudine alone, adefovir alone, lamivudine plus adefovir, or left untreated. The HBV DNA replicative intermediate in the cytoplasmic fraction of the cells was detected by Southern blot analysis. A: Representative result of Southern blot analysis to detect the HBV DNA replicative intermediate. SS, single-stranded HBV DNA. DL, double-stranded linear HBV DNA. RC, relaxed circular HBV DNA. B: Quantitative analysis of the HBV DNA replicative intermediate in cells transfected with pHBC, pHBC-C1753 and pHBC-C2189 without nucleos(t)ide analog treatment. The level of the HBV DNA replicative intermediate in the case of transfection with pHBC was considered as 1, and its fold activity in the case of transfection with the mutant HBV-expressing plasmid was calculated. The experiment was done three times, and the results are presented as the mean ± SD. *$P < 0.001$ versus pHBC and pHBC-C1753 groups. C: Degree of reduction in the HBV DNA replicative intermediate after treatment with lamivudine and/or adefovir in cells transfected with pHBC, pHBC-C1753 and pHBC-C2189. The level of the HBV DNA replicative intermediate in untreated cells was considered as 1, and its fold activity in cells treated with lamivudine and/or adefovir was calculated. The experiment was done three times, and the results are presented as the mean ± SD.

regimen for lamivudine-resistant patients with type B chronic hepatitis. In the present study, the viral mutations associated with the effect of this regimen were investigated by screening the whole HBV genome via sequencing analysis of full-length viral DNA. Two mutations, V1753 and C2189, were identified as significant determinants of the therapeutic efficacy. Using adefovir dipivoxil added to lamivudine treatment, HBV DNA tended to decline to the undetectable level more frequently in patients with the V1753 or C2189 mutation than in those without it. In univariate analysis, only the presence of the V1753 or C2189 mutation was shown to be a factor contributing to sustained clearance of HBV DNA during adefovir dipivoxil therapy. Multivariate analysis also revealed that the V1753 and C2189 mutations, as well as high ALT and low HBV DNA at baseline, were independent factors associated with a better antiviral effect. Reports from the United States and European countries have revealed that female gender, high ALT, low viral load, absence of HBeAg and genotype D rather than genotype A were related to a better outcome of adefovir dipivoxil therapy in nucleoside-naïve and lamivudine-resistant patients with type B chronic hepatitis [Lampertico et al., 2005; Fung et al., 2006; Buti et al., 2007]. The findings of the present study from Japan, a genotype C HBV-endemic area, agreed in part with these reports. Of particular interest is the finding that the therapeutic efficacy of adefovir dipivoxil added to lamivudine may be affected not only by clinical factors but also the genomic background of HBV such as the presence of the V1753 or C2189 mutation in lamivudine-resistant patients with type B chronic hepatitis. In addition, serial sequencing analysis revealed that both the V1753 and C2189 mutations tended to be selected during lamivudine therapy associated with the establishment of lamivudine resistance, although they have been shown to be mutations which occur naturally during the course of HBV infection [Ehata et al., 1991; Bozkaya et al., 1996; Takahashi et al., 1999; Imamura et al., 2003; Ozasa et al., 2006; Tanaka et al., 2006].

The findings of the present study suggest higher sensitivity to adefovir dipivoxil therapy of the V1753 and C2189 mutant viruses compared to the wild-type virus in vivo. However, in vitro transfection analysis showed no differences in susceptibility to adefovir, as well as to lamivudine, among the wild-type virus and the C1753 and C2189 mutant viruses. This indicates that the V1753 and C2189 mutant viruses may be eradicated more efficiently by adefovir dipivoxil therapy than the wild-type virus regardless of a direct antiviral effect of adefovir dipivoxil. The V1753 and C2189 mutant viruses may induce stronger immune responses against the viral pathogens than the wild-type virus, which might result in more frequent viral eradication under adefovir dipivoxil therapy in patients having the V1753 or C2189 mutant virus compared to those with the wild-type virus.

Of the 1421 HBV strains, whose nucleotide sequences of the BCP, precore and core regions had been identified and registered in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp), there were 259 (18%) strains with the V1753 mutation and 127 (9%) strains with the C2189 mutation. The V1753 mutation was found in strains of all HBV genotypes, whereas the C2189 mutation was found in strains of genotypes A, B, C, and E. Thus, the V1753 and C2189 mutations were not specific for genotype C but common in other HBV genotypes.

The V1753 mutation occurring in the BCP not only influences the core promoter activity but also causes the I127T/N/S amino acid change of the overlapping X gene. This mutation has been detected in a considerable proportion of chronic HBV carriers, especially coupled with the adjacent T1762/A1764 mutation [Kidd-Ljunggren et al., 1997; Takahashi et al., 1999]. Indeed, all 11 patients with the V1753 mutation possessed the T1762/A1764 mutation in the current study. It has also been shown that, among patients with type B chronic hepatitis of genotype C, the V1753 mutation was found more frequently in patients with HCC than in those without it [Tanaka et al., 2006]. In acute HBV infection, the frequency of mutation has been reported to be higher in patients with fulminant hepatitis than in those with non-fulminant hepatitis [Imamura et al., 2003; Ozasa et al., 2006]. In vitro transfection assay revealed that the C1753 mutant virus possessed similar replicative competence to the wild-type virus, though viruses having the G1753 and A1753 mutation were not examined. Also, the in vitro replicative competence did not differ between the wild-type and C1753 mutant viruses when the T1762/A1764 mutation was introduced into the backbone HBV structure (data not shown). According to these observations, the serious disease course and better response to adefovir dipivoxil therapy caused by the V1753 mutation, as suggested by the present study and other previous investigations [Imamura et al., 2003; Ozasa et al., 2006; Tanaka et al., 2006], may not be due to the modification of the viral replicative competence. Further studies should be done to clarify why the V1753 mutation is involved in the active liver disease and the better outcome of adefovir dipivoxil therapy in patients with HBV infection.

The C2189 mutation, which leads to the I97L amino acid change in the core gene, has also been shown to be detected frequently in patients with type B chronic hepatitis [Ehata et al., 1991; Bozkaya et al., 1996], although the relevance of the mutation to a particular disease course has not been elucidated fully. Previous in vitro transfection studies have suggested that the virus with the C2189 mutation resulted in excessive secretion of the immature virion and enhanced viral replication [Yuan et al., 1999; Suk et al., 2002]. This does not agree with the present result showing lower replicative competence of the C2189 mutant virus than the wild-type virus. This discrepancy may be due to the usage of HBV-expressing plasmids of different viral strains. The virological and clinical significance of the C2189 mutant virus should be assessed by further detailed investigation.

In summary, the results of the present study indicate that the presence of the two viral mutations, V1753 and C2189, may be associated with a better therapeutic effect of adefovir dipivoxil added to lamivudine based on the results of screening of the full-length HBV genome obtained from lamivudine-resistant patients with type B chronic hepatitis. As the present study examined a limited number of patients with HBV of genotype C, further studies with a larger number of patients with different genotypes should lead to a better understanding of how identifying these mutations can be useful in a clinical setting.

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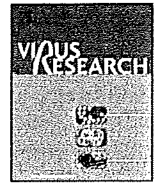

Accumulation of LANA at nuclear matrix fraction is important for Kaposi's sarcoma-associated herpesvirus replication in latency

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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.

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1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) is a gamma-herpesvirus 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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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 $10,000 \times g$ 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 $10,000 \times g$ 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 $10,000 \times g$ 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).

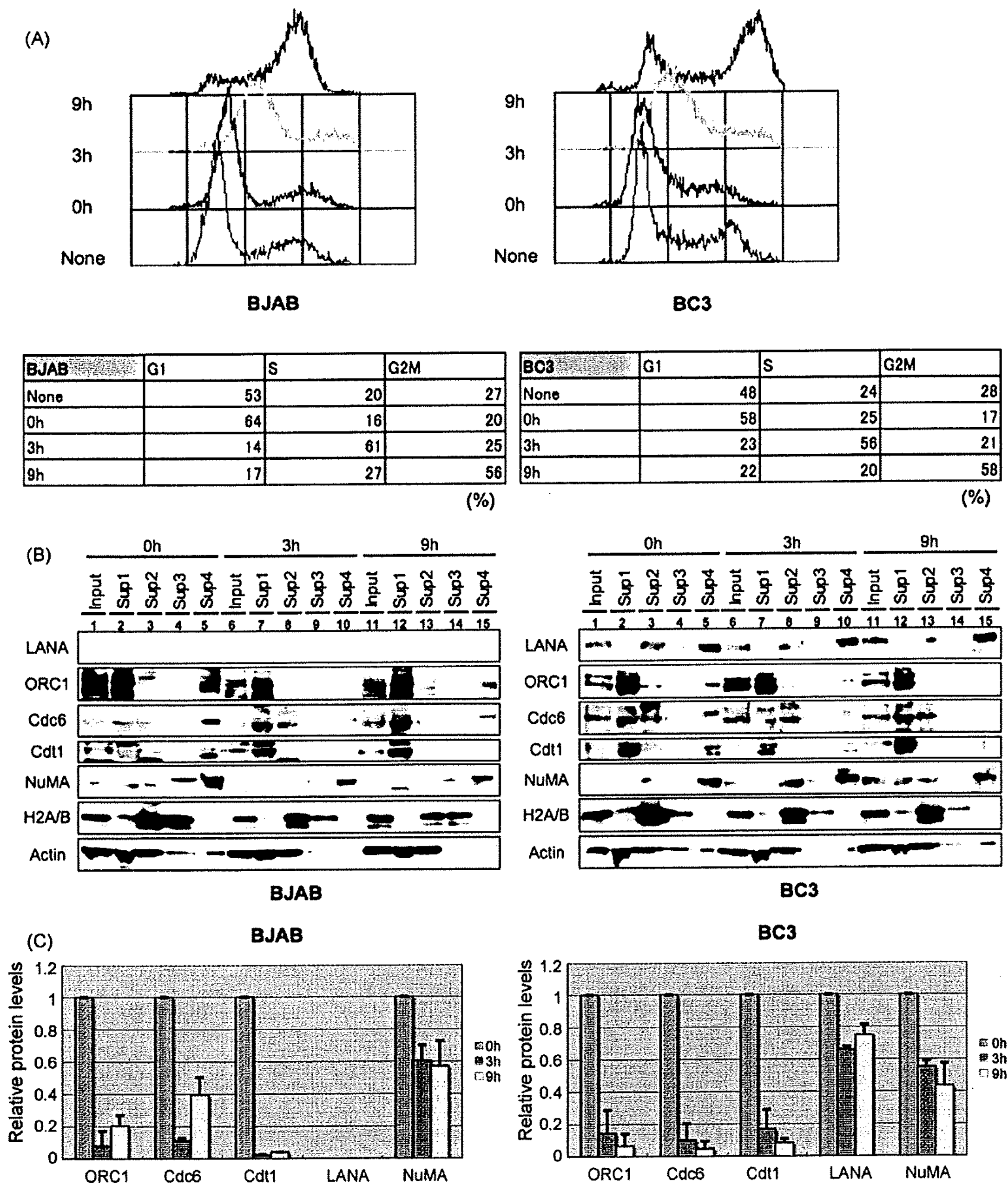
3. Results

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

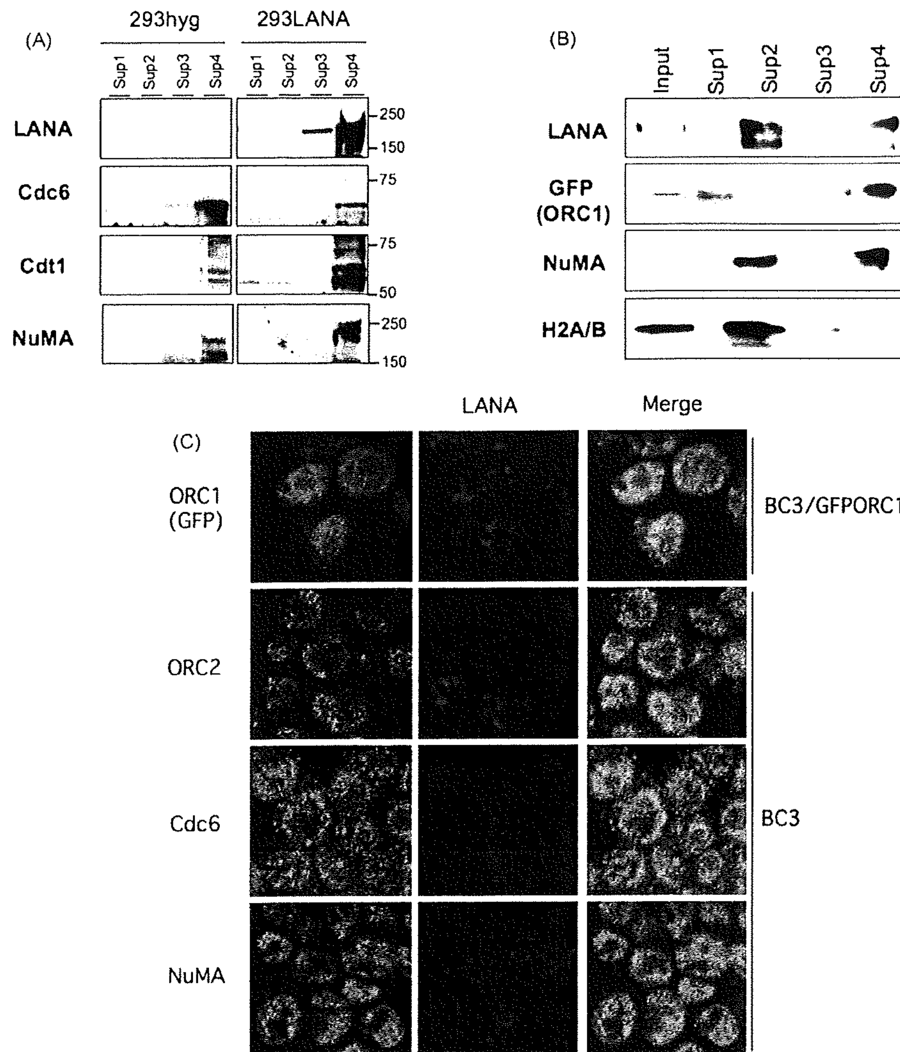


Fig. 2. LANA self-associates with the nuclear matrix fraction and colocalizes with host pre-RC components. (A) One million 293hyg or 293LANA cells were harvested and fractionated. Ten percent of each fraction was separated on 10% SDS-PAGE and subjected to Western blotting analysis. (B) One million GFP-ORC1/BC3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

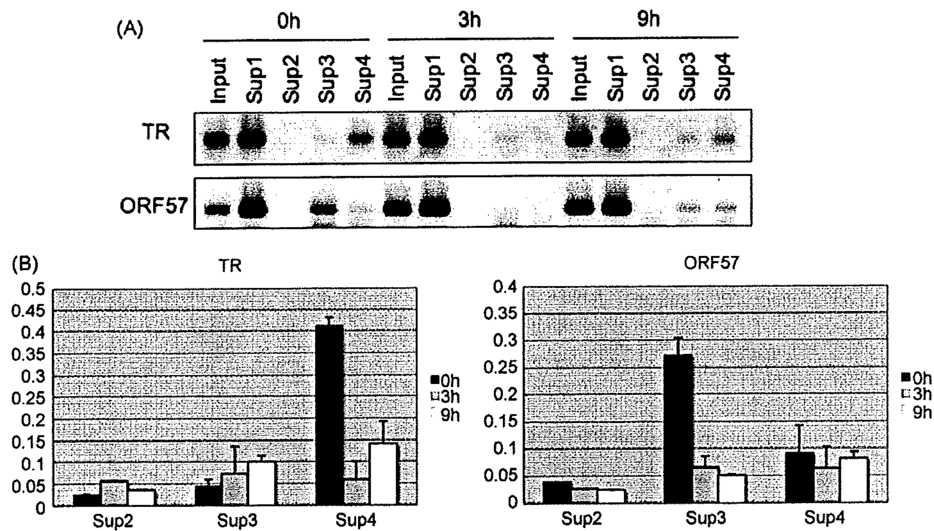


Fig. 3. The TR region (ori-P) is present in the nuclear matrix in a cell-cycle-dependent manner. DNA from fractions of the BC3 cells prepared in Fig. 1C was extracted as described in Section 2. The amount of DNA was measured by spectrophotometer (DU640, Beckman) and was adjusted to 10 ng/μl. Input DNA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

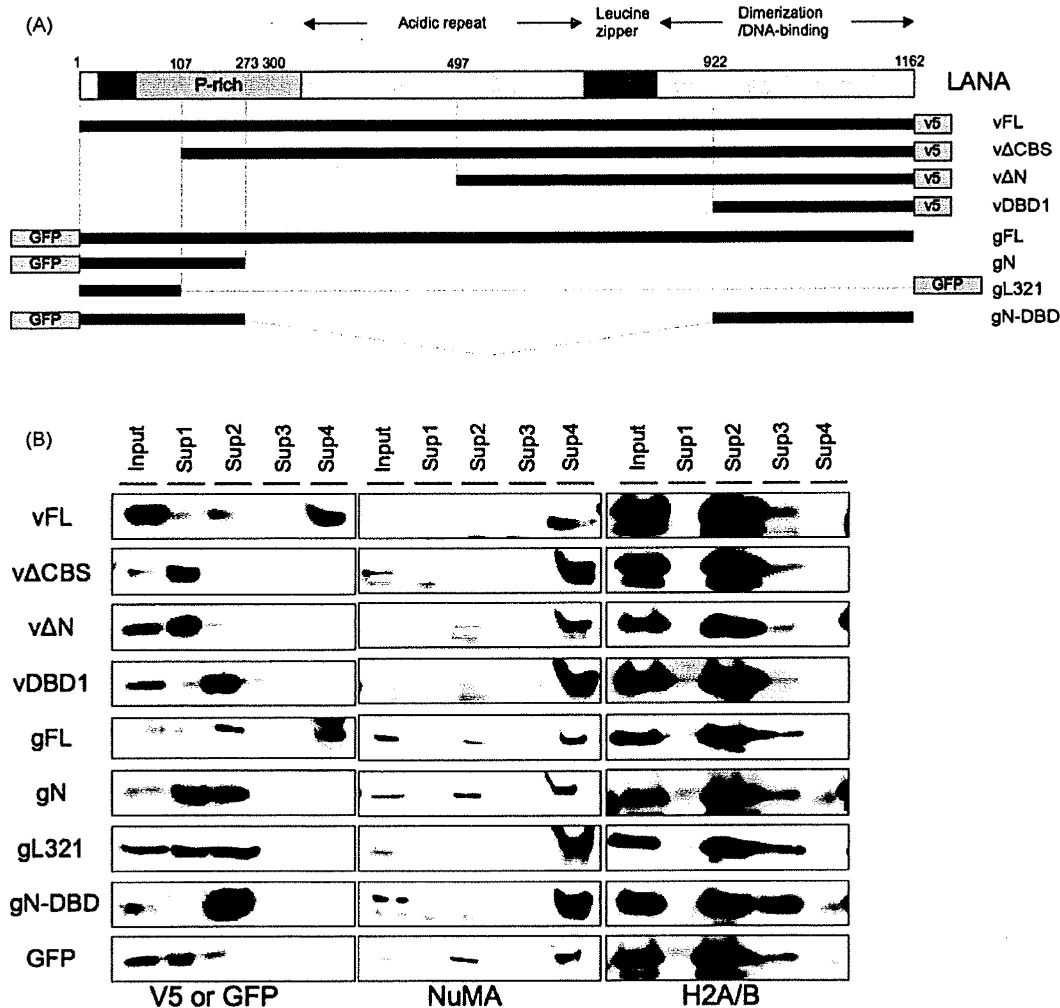


Fig. 4. Nuclear localization of LANA is required for ori-P mediated viral replication. (A) Schematic presentation of LANA expression constructs. Two full-length LANA expression vector was constructed. One was tagged at the C-terminus with a V5 epitope (vFL) and another was at the N-terminus with a GFP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

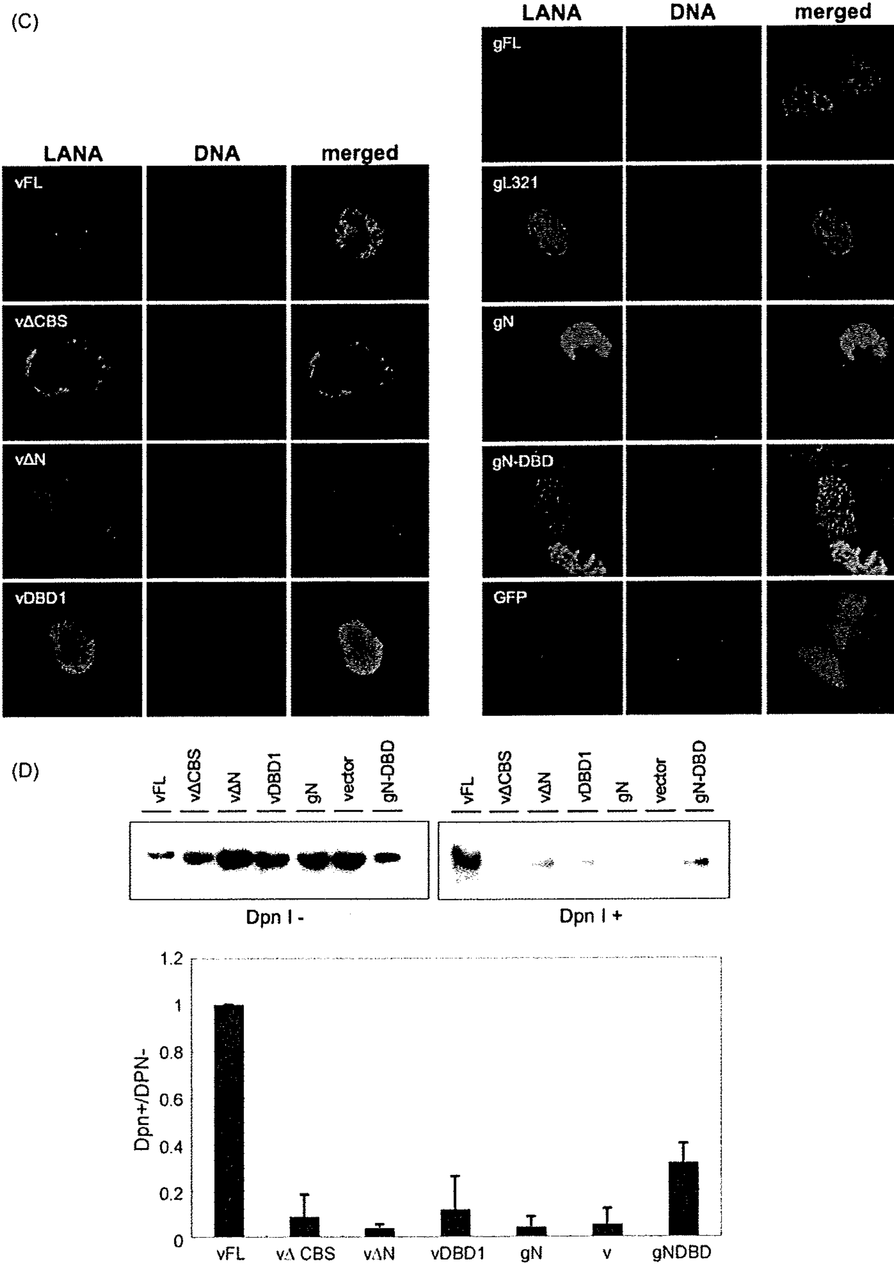


Fig. 4. (*Continued*).

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

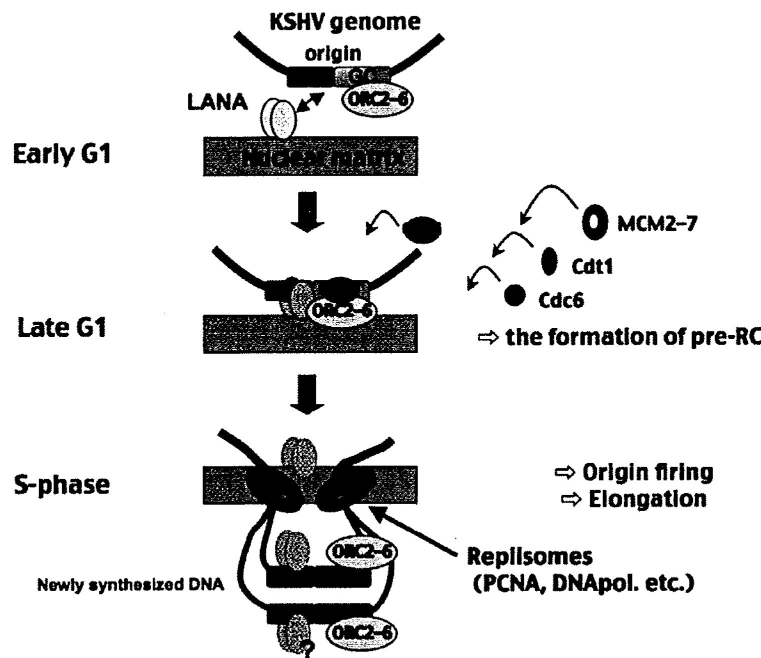


Fig. 5. A suggestive model for replication of the KSHV genome at the nuclear matrix. LANA associates with the nuclear matrix in itself and recruits the ori-P of KSHV genome to the nuclear matrix region by binding to the LBS. The complete pre-RC is formed on the nuclear matrix in the late G1 phase. In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

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