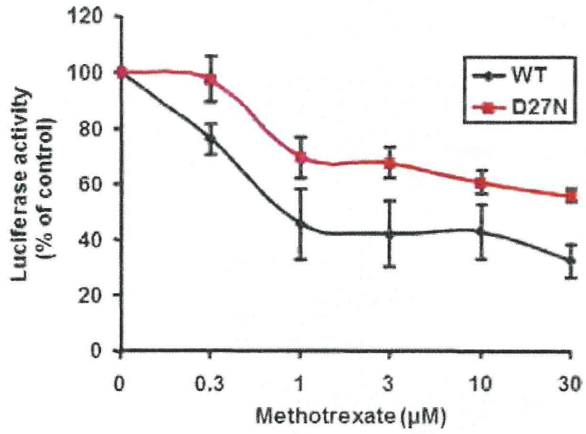


**Fig. 3.** Methotrexate-resistance of D27N mutant by mini-replicon assay system. The mini-replicon assay using *luciferase* gene as a reporter gene was carried out. At 12 hpt, different concentrations (0, 0.3, 1, 3, 10, and 30 μM) of methotrexate were added, and luciferase activity was measured at 22 hpt. The vertical axis represents the percentage of the luciferase activity from methotrexate-treated cells relative to that from methotrexate-untreated cells. The results are averages from three independent experiments with standard deviations.

viral RNA synthesis. We found that the viral polymerase activity of D27N was higher than that of wild type in the presence of ribavirin (Fig. 2C). Further, the viral polymerase activity of D27N was also resistant to MTX treatment compared with that of wild type (Fig. 3). These strongly suggest that D27 N mutant can polymerize the nascent RNA chains with the low concentrations of nucleotide. Therefore, it is expected that Asp27 is involved in the nucleotide recognition.

The Asp27 of PB1 is conserved over 99.9% of 7259 sequences of PB1 deposited in the NCBI Influenza Sequence Database. It has been reported that Asp27 is located upstream of the nucleotide binding site of PB1 but not in the catalytic active site. Similarly, the ribavirin-resistant mutant of poliovirus has a mutation in a domain out of the catalytic active site of the viral polymerase [42]. D27N is present within putative vRNA and cRNA promoter binding sites [25,27]. It is shown by mutants in the promoters of vRNA and cRNA that the RNA synthesis activity, cleavage of the cap structure, and the polyadenylation by viral polymerase are regulated through the promoter structure [43–45]. Further, the viral polymerase is stabilized by the interaction with its viral promoter [46]. Based on previous reports and our findings, the interaction between Asp27 of PB1 with vRNA and/or cRNA promoters may lead to the regulation of viral polymerase activity through the nucleotide recognition activity of PB1. This finding could useful for further studies about the mechanism of nucleotide recognition of the influenza viral RNA polymerase.

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# The N-terminal region of influenza virus polymerase PB1 adjacent to the PA binding site is involved in replication but not transcription of the viral genome

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The influenza virus genome forms viral ribonucleoprotein (vRNP) complexes with nucleoprotein and viral RNA-dependent RNA polymerases (RdRp), PB1, PB2, and PA subunits. The vRNP complex catalyzes both genome replication and transcription reactions. PB1 contains the motifs highly conserved among RdRps and functions as a catalytic subunit of RdRp. The N-terminal region of PB1 between amino acid (a.a.) positions 1–83 contains both putative vRNA and cRNA promoter binding sites and a PA binding site. However, except for the PA binding site, the crystal structure and the function of the N-terminal region of PB1 are poorly understood. Here, we have examined the functional structure of the N-terminal region of PB1. The regions between a.a. positions 1–50 are highly conserved between influenza A and B viruses, but amino acids at positions 16, 27, and 44 are different between two viruses. To elucidate the functional importance of these amino acids in replication and transcription of the viral genome, we generated viruses containing mutations at these positions by reverse genetics and examined replication and transcription activities of these mutants. We found that a.a. positions 27 and 44 are responsible for the viral replication activity but not transcription activity.

**Keywords: influenza virus, promoter binding, replication, reverse-genetics, RNA-dependent RNA polymerase, transcription**

## INTRODUCTION

Influenza A and B viruses contain eight-segmented and negative-stranded RNAs (vRNA) as its genome. Each segment is encapsidated by nucleoprotein (NP) and associated with viral RNA-dependent RNA polymerases (RdRp) to form viral ribonucleoprotein (vRNP) complexes. The vRNP complex is a basic unit for both genome replication and transcription (Nagata et al., 2008).

The viral RdRp is a heterotrimer consisting of PB1, PB2, and PA subunits. Among them, PB1 functions as a catalytic subunit and assembly core of RdRp (Biswas and Nayak, 1996; Gonzalez et al., 1996; Toyoda et al., 1996; Zurcher et al., 1996; Ohtsu et al., 2002). The crystal structure of the interaction domains between N-terminal region of PB1 and C-terminal region of PA, and between C-terminal region of PB1 and N-terminal region of PB2 were resolved (He et al., 2008; Obayashi et al., 2008). PB1 contains the motifs highly conserved among RdRps, putative nucleotide-binding sites, and vRNA and cRNA promoter binding sites (Asano and Ishihama, 1997; Li et al., 1998; Gonzalez and Ortin, 1999a,b; Kolpashchikov et al., 2004) (**Figure 1A**).

The N-terminal region of PB1 (1–83 a.a.) contains both putative vRNA and cRNA promoter binding sites. However, except for the PA binding site (1–15 a.a.), the function of this region was poorly understood. Alignment of amino acid sequences revealved that the a.a. positions 1–50 was highly conserved between influenza A and B viruses except for the amino acid positions 16, 27, and 44. To identify the functional importance of these positions for the viral RNA synthesis, we determined the replicational and transcriptional activities using mutant viruses. Our results strongly suggest that the a.a. positions 27 and 44 are involved in replication process but not transcription process.

## MATERIALS AND METHODS

### BIOLOGICAL MATERIALS

Monolayer cultures of 293T and MDCK cells were maintained at 37°C with 5% $CO_2$ in Dulbecco's Modified Eagle Medium (DMEM) and minimal essential medium (MEM) (Nissui), respectively, supplemented with 10% fetal bovine serum (Bovogen). Influenza virus strain A/WSN/33 (WSN) was prepared as previously described (Kawaguchi et al., 2005). Cycloheximide (CHX) was purchased from Sigma-Aldrich.

### GENERATION OF RECOMBINANT VIRUSES

To construct plasmids from which human DNA-dependent RNA polymerase I (Pol I) transcribes mutated vRNAs, we amplified fragments containing mutated segment 2 by PCR using a plasmid containing wild type WSN segments in pHH21 vector (Neumann et al., 1999) as a template with sets of phosphorylated primers (see Table S1 in the supplemental material). The amplified PCR products were self-ligated followed by sequencing. To generate recombinant viruses containing viral RNAs of WSN and mutated segment 2, reverse genetics system was used as described previously (Neumann et al., 1999). After 48 h post transfection (hpt), aliquots of cell culture supernatants were used for virus

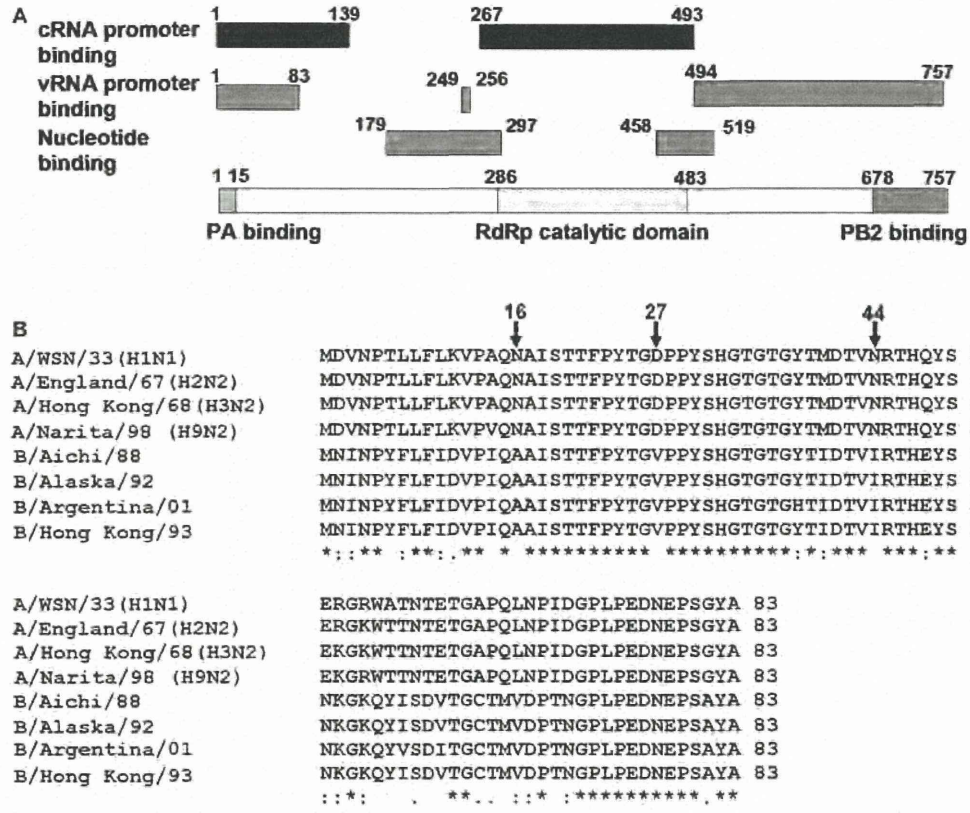


**FIGURE 1 | Structure of PB1 subunit. (A)** A diagrammatic representation of PB1. Black bar, cRNA promoter binding sites; gray bar, vRNA promoter binding sites; black and white vertical stripes, nucleotide binding sites; black and white horizontal stripes, PB2 binding site; black waved lines, PA binding site, and black dots, RNA dependent-RNA polymerase catalytic domain. **(B)** Alignment of amino acid sequences of putative RNA binding region common to vRNA and cRNA promoters (1–83 a.a.) among influenza A and influenza B viruses. PB1 sequences of A/WSN/33, A/WSN/1933 (H1N1); A/England/67, A/England/10/67 (H2N2); A/Hong Kong/68, A/Hong Kong/1/1968 (H3N2); A/Narita/98, A/parakeet/Narita/92A/98 (H9N2). These strains are picked up from different clades and periods of PB1 gene phylogenetic tree (Taubenberger et al., 2005); B/Aichi/88, B/Aichi/5/88; B/Alaska/92, B/Alaska/03/1992; B/Argentina/01, B/Argentina/132/2001; and B/Hong Kong/93, B/Hong Kong/02/1993. Sequences were aligned with CLUSTAL W. Asterisk (*), identical residues; colon (:), conserved residues, and dot (.), semi-conserved residues.

amplification in MDCK cells. At 48 h post infection (hpi), the culture fluid was collected, and the virus titer of these recombinant viruses was determined by plaque assays.

### RNA ANALYSIS BY qRT-PCR

MDCK cells were infected with recombinant viruses at the multiplicity of infection (MOI) of 2.5. At 9 hpi, total RNA was isolated by the acid guanidine-phenol-chloroform method. To measure the accumulation levels of viral mRNA, cRNA, and vRNA, quantitative RT-PCR (qRT-PCR) was performed. Total RNAs were subjected to reverse transcription using ReverTraAce (Toyobo) with either (i) oligo $(dT)_{20}$, (ii) 5′-AGTAGAAACAAGGGTATTTTTCTTTA-3′, or (iii) 5′-GACGATGCAACGGCTGGTCTG-3′ for synthesizing cDNA from segment 5 mRNA, cRNA, and vRNA, respectively (Kawaguchi and Nagata, 2007; Sugiyama et al., 2009). The synthesized single-stranded cDNAs were subjected to real-time quantitative PCR analysis (Thermal Cycler Dice real-time system TP800; TaKaRa) with SYBR Premix Ex *Taq* (TaKaRa) and a set of specific primers for segment 5 cDNA (see supplementary methods). The levels of these RNAs were normalized by the amount of cellular β-actin mRNA measured using specific primers (see supplementary methods). These results are averages from three independent experiments with standard deviations. The level of significance was determined by Student's *t*-test (unpaired).

## RESULTS

### RNA SYNTHESIS OF INFLUENZA A MUTANT VIRUSES CONTAINING INFLUENZA B VIRUS-TYPE AMINO ACID SIGNATURES

The N-terminal region of PB1 (1–83 a.a.) contains the PA binding site and both putative vRNA and cRNA promoter binding sites (**Figure 1A**). It is shown by alignment of amino acid sequences that the PB1 region between a.a. positions 1–50 are highly conserved between influenza A and B viruses, while the region between a.a. positions 51–83 differ between two viruses (**Figure 1B**). In the highly conserved region, except for the PA binding site, a.a. at positions 16, 27, and 44 are different between these viruses. Furthermore, these a.a. positions in PB1 are conserved more than 99% of influenza A and B viruses deposited in the NCBI Influenza Virus Sequence Database (**Table 1**). To elucidate the functional importance of these a.a. for viral RNA

**Table 1 | Conservation of amino acid position 16, 27, and 44 in PB1.**

| Virus type | a.a. position | Mutation | No. of strains (total strains) | Percentage |
|---|---|---|---|---|
| **INFLUENZA A VIRUS** | | | | |
| | 16 | N (wild type) | 7228 (7259) | 99.6% |
| | | S | 24 (7259) | 0.3% |
| | | D | 4 (7259) | 0.1% |
| | | K | 1 (7259) | 0.0% |
| | | H | 1 (7259) | 0.0% |
| | | Y | 1 (7259) | 0.0% |
| | 27 | D (wild type) | 7250 (7259) | 99.9% |
| | | N | 4 (7259) | 0.1% |
| | | E | 3 (7259) | 0.0% |
| | | G | 2 (7259) | 0.0% |
| | 44 | N (wild type) | 7204 (7259) | 99.2% |
| | | S | 42 (7259) | 0.6% |
| | | T | 11 (7259) | 0.2% |
| | | D | 2 (7259) | 0.0% |
| **INFLUENZA B VIRUS** | | | | |
| | 16 | A (wild type) | 1412 (1412) | 100% |
| | 27 | V (wild type) | 1412 (1412) | 100% |
| | 44 | I (wild type) | 1409 (1412) | 99.8% |
| | | V | 3 (1412) | 0.2% |

*The conservation of amino acid position 16, 27, and 44 in PB1 was calculated by using 7259 sequences of human and avian influenza A strains and 1412 sequences of influenza B strains listed at the NCBI Influenza Sequence Database.*

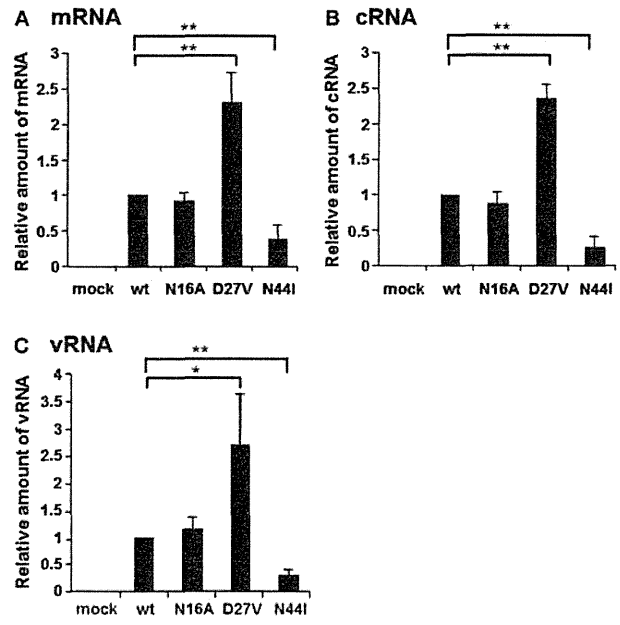


**FIGURE 2 | Comparison of RNA synthesis of mutant influenza A viruses containing amino acids specific for influenza B viruses.** The amino acids, N, D, and N at the positions 16, 27, and 44 were mutated to A, V, and I, respectively. MDCK cells were infected with mutant viruses at MOI of 2.5. At 9 hpi, the accumulation levels of viral mRNA **(A)**, cRNA **(B)**, and vRNA **(C)** were measured by qRT-PCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations. $*p < 0.05$; $**p < 0.01$.

synthesis, we generated influenza A viruses containing Ala at the a.a. position 16 (N16A), Val at the a.a. position 27 (D27V), and Ile at the a.a. position 44 (N44I) by reverse genetics. We examined the RdRp activity by measuring the accumulation levels of viral mRNA, cRNA, and vRNA by qRT-PCR (**Figure 2**). The levels of all three type RNAs from D27V were increased compared with those from wild type and N16A virus, while those from N44I were significantly decreased. Based on the result that mutations at the positions 27 and 44 affect the synthesis activity of all viral RNAs equally, there could be two possibilities: (i) these mutations affect on the vRNA promoter recognition and followed by cRNA/mRNA synthesis, but do not affect on the cRNA promoter recognition and followed by vRNA synthesis, or (ii) these mutations affect independently the synthesis of each viral RNA, but total effects leads similar outputs in the synthesis of all viral RNAs.

To elucidate whether these mutations affect genome replication (cRNA and vRNA synthesis) and/or transcription (viral mRNA synthesis) activities, we measured the primary transcription activity using cycloheximide (CHX), a potent inhibitor of protein synthesis (**Figure 3**). It is shown that CHX suppresses viral protein synthesis and thereby leads to degradation of replicated virus genome RNA but not viral mRNA since newly vRNP formation was repressed (Vreede et al., 2004; Kawaguchi et al., 2005). We utilized this method to measure the primary transcription activity that depends just only on incoming vRNP and is not affected by the replication process. In the presence of CHX, the levels of viral mRNA and vRNA were measured by qRT-PCR, and the transcription activity was represented as a ratio of viral mRNA/vRNA. This result shows that the transcription activity is not affected by these mutations, and thereby strongly suggests that these mutations affect the replication activity.

## AMINO ACID PROPERTIES AT a.a. POSITIONS 27 AND 44 FOR THE RNA SYNTHESIS ACTIVITY

Aspartate at the position 27 is highly conserved among influenza A viruses, except for an H4N8 strain isolated from least sandpiper that contains asparagine (GenBank: ACI90144.1). We generated D27E and D27N in addition to D27V (**Figure 4**). The RNA levels of mRNA, cRNA, and vRNA of D27N and D27V were increased significantly compared with those of wild-type and D27E. One of possible interpretations is that uncharged amino acids at a.a. position 27 may enhance the RNA synthesis.

The mutation at the a.a. position 44 reduced the replication activity (**Figure 2**). To clarify the importance of the a.a. at this position, we additionally generated N44D and N44Q viruses in addition to N44I and examined the RNA synthesis activity (**Figure 5**). The synthesis level of each viral RNA of N44I was decreased largely, while the amounts of mRNA and cRNA of N44D and N44Q were similar to those of wild type. In addition, the amount of vRNA of N44Q was more than that of wild type.

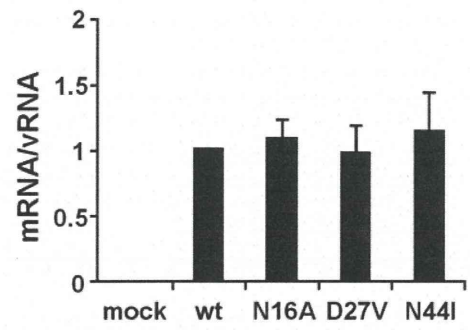


**FIGURE 3 | Primary transcription activity of mutant influenza A viruses containing amino acids specific to influenza B viruses.** MDCK cells were infected with mutant viruses at MOI of 2.5 and incubated in the presence of 1.0 μg/ml of CHX. At 9 hpi, the accumulation levels of viral mRNA and vRNA were measured by qRT-PCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. The transcription activity is represented as a ratio of the amount of viral mRNA to that of vRNA. These results are averages from three independent experiments with standard deviations.

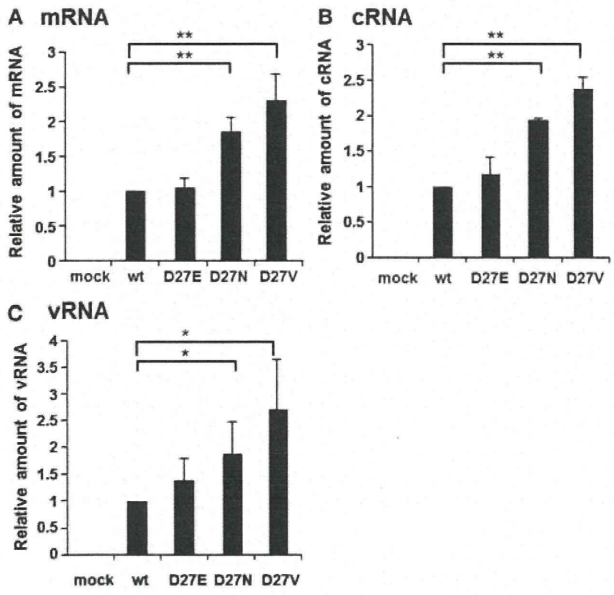


**FIGURE 4 | RNA synthesis of viruses containing mutations at the amino acid position 27.** Wild-type virus and mutant viruses containing amino acids D and E, N, and V, respectively, at the a.a. position 27 were infected into MDCK cells at MOI of 2.5. At 9 hpi, the accumulation levels of viral mRNA **(A)**, cRNA **(B)**, and vRNA **(C)** were measured by qRT-PCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations. $*p < 0.05$; $**p < 0.01$.

Thus, it is expected that the a.a. position 44 might be a water-soluble characteristic, and especially glutamine at this position stimulates the vRNA synthesis.

## DISCUSSION

In this report, we have studied on three a.a. positions, i. e., 16, 27, and 44, which are not conserved between influenza A and B

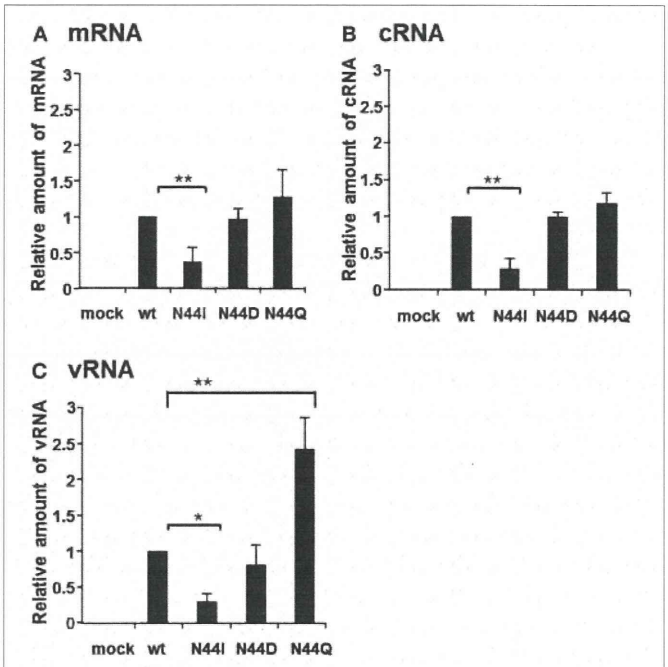


**FIGURE 5 | RNA synthesis of viruses containing mutations at the amino acid position 44.** Wild-type virus and mutant viruses containing amino acids N and I, D, and Q, respectively, at the a.a. position 44 were infected into MDCK cells at MOI of 2.5. At 9 hpi, the accumulation levels of viral mRNA **(A)**, cRNA **(B)**, and vRNA **(C)** were measured by qRT-PCR, and the amounts of these RNAs were normalized by that of cellular actin mRNA. These results are averages from three independent experiments with standard deviations. $*p < 0.05$; $**p < 0.01$.

viruses. The RNA synthesis activity of D27V was enhanced, while that of N44I was decreased (**Figure 2**). Based on these, we carried out further mutational analyses. The N44I showed the decreased level of RNA synthesis in three types of viral RNAs, while N44D did not affect the RNA synthesis (**Figure 5**). Interestingly, N44Q increased vRNA synthesis with little effect on viral mRNA and cRNA synthesis. It is possible that side chain group of Q may stimulate the cRNA promoter binding and increase the vRNA synthesis activity.

D27V and D27N increased the RNA synthesis, while D27E mutation gave no effects (**Figure 4**). Furthermore, when the amounts of RNAs of D27V were analyzed at various MOI, those of D27V were increased (Figure S3). Although uncharged amino acid at this position enhances the RNA synthesis, molecular evolution has selected negatively charged amino acids. Therefore, it is assumed that charged amino acids at this position, even with low efficiency for the replication, are needed for PB1. Recently, mutational analyses showed that the sequences surrounding the PB1 AUG codon are multifunctional, and contain overlapping signals for translation initiation and for segment specific packaging (Wise et al., 2011). We may consider a possibility that there is some regulatory coupling between replication and packaging and the a.a. position 27 has a role in this hypothetical mechanism.

These a.a. positions are close to the PA binding site, and the PB1-RNA interaction could be affected by the presence of PA. We examined whether these mutations affect the assembly of

RdRp (Figure S1). The assembly of PB1 with PA and PB2 was not affected by these mutations. Moreover, these mutations did not affect the transcription activity, mRNA synthesis from vRNA (**Figure 3** and Figure S2). Taken altogether, it is quite likely that amino acids at the positions 27 and 44 are involved in the replication activity, possibly in cRNA promoter recognition with little effects on the transcription activity and the assembly of the RdRp complex.

Recognition of the vRNA promoter depends on the 5′-arm of the promoter and this binding improves the weak binding of RdRp to the 3′-arm of the vRNA promoter (Tiley et al., 1994; Gonzalez and Ortin, 1999a; Jung and Brownlee, 2006). Recognition of the cRNA promoter by RdRp has been shown by the *in vitro* binding of the PB1 subunit with the 5′- and 3′-arms of the cRNA promoter (Gonzalez and Ortin, 1999b). Flexibility within the two uridines of the internal loop of the cRNA promoter required for protein binding in the cRNP complex (Park et al., 2003). Biochemical studies have shown that conformational changes in PB1 of the influenza A virus RdRp lead to the interaction with either vRNA or cRNA (Gonzalez and Ortin, 1999b). Based on previous reports and our findings, the positions 27 and 44 may affect the PB1 structure, resulting in affecting PB1 binding activity to the 3′-arm of the cRNA promoter. Thus, we would propose that these positions in PB1 are important for the replication activity by recognizing the cRNA promoter.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2013.00398/abstract

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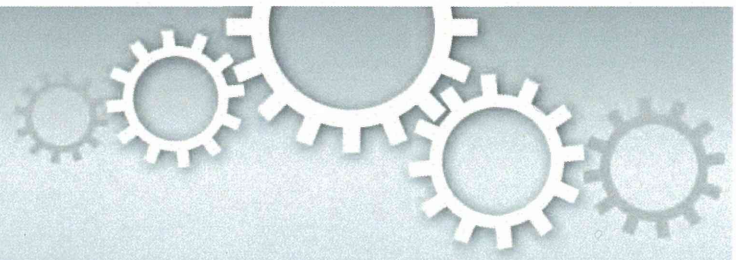

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# DNA replication-dependent binding of CTCF plays a critical role in adenovirus genome functions

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**The expression of adenovirus late genes is shown to require viral DNA replication, but its mechanism remains elusive. Here we found that knockdown of CTCF suppresses viral DNA replication as well as late, but not early, gene expression. Chromatin immunoprecipitation assays indicated that CTCF binds to viral chromatin depending on viral DNA replication. These findings depict CTCF as a critical regulator for adenovirus genome functions in late phases of infection.**

In the cell nucleus, the genomic DNA forms chromatin structure. It is being clarified that the higher-order chromatin structure, such as the DNA looping, plays an important role in a dynamic property of the chromatin[1]. One of the chromatin organizing proteins, CTCF (CCCTC-binding factor), is a well-characterized chromatin-binding factor involved in the formation of the long-range interactions of chromatin[2]. CTCF has eleven zinc fingers and therefore binds to divergent DNA sequences, as indicated by chromatin immunoprecipitation (ChIP) in combination with tiling arrays (ChIP-on-chip)[3] or high-throughput sequencing analyses (ChIP-seq)[4]. A variety of chromatin-related proteins are reported as binding partners of CTCF, including cohesin complexes[5,6], a nucleolar protein B23/nucleophosmin, and CTCF itself[7]. These interactions are thought to enable CTCF binding sites to contact each other and/or be tethered to the subnuclear domains, resulting in the formation of intra- and interchromatin interaction[2]. In addition to the role on the cellular chromatin, recent reports have revealed the involvement of CTCF on viral proliferation, as Lieberman and co-workers recently demonstrated the CTCF-mediated formation of chromatin loops on Kaposi's sarcoma-associated Herpesvirus (KSHV) and Epstein-Barr virus (EBV) genomes[8,9]. It is shown that CTCF regulates the latency-specific chromatin conformation of KSHV and EBV genomes, and siRNA-mediated depletion of CTCF or mutations in the CTCF binding sites disrupt the chromatin architecture and de-regulate latent gene expression[8,9]. Thus, CTCF could impact on the regulation of not only cellular but also viral chromatin.

The adenovirus (Ad) has a linear double-stranded DNA genome that forms chromatin-like structure in the virion[10]. Previously, we have reported that viral chromatin structure regulates the expression of viral early genes (e.g. E1A, E4 genes) in early phases of infection[11,12]. The expression of the late genes (e.g. major late genes) are hardly observed during early phases of infection, while concomitantly with the onset of viral DNA replication, those genes are fully activated. Thomas and Mathews demonstrated that the expression of the late genes requires viral DNA replication in *cis*[13]. In addition, we have shown the regulatory mechanism of the viral chromatin structure during DNA replication and proposed a possible role of viral DNA replication in the activation of late genes[14]. Thus, it is suggested that the regulation of viral chromatin structure has a significant role in the DNA replication-dependent activation of viral genes. In spite of these evidences, however, the functional relationship between viral gene expression and DNA replication in infected cells remains largely unclear. In this study we sought to further clarify the role of chromatin structure and/or chromatin-related factors on the Ad genome DNA. As described above, it is shown that CTCF plays a role on the chromatin of some DNA viruses[8,9]. These lead us to hypothesize that CTCF could function also on Ad chromatin.

## Results

**CTCF is required for viral DNA replication and late gene expression.** To study a role of CTCF, we carried out knock down (KD) of the expression of CTCF by siRNA treatment (Fig. 1A). Either control siRNA (siCont) or

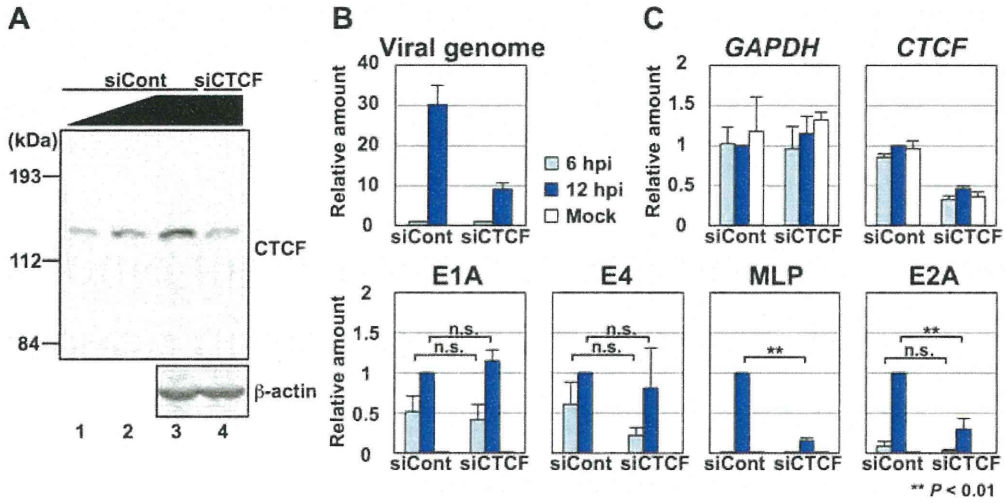


**Figure 1 | Effects of CTCF KD on viral DNA replication and gene expression.** (A) Western blotting and knockdown of CTCF. Cell lysates were prepared from HeLa cells treated with siCont (lanes 3) or siCTCF (lane 4) and subjected to western blot analyses using anti-CTCF (top panel) and anti-β-actin antibodies (bottom panel). For siCont-treated cells, 25% (lane 1) and 50% (lane 2) volume of lysates were also loaded. Full-size images are shown in Supplementary figure 1. (B) Amounts of viral DNA. HeLa cells treated with siCont or siCTCF were infected with HAdV5 at an MOI of 100, and total DNAs were purified at 6 and 12 hpi. The amount of viral DNA was measured by qPCR using primers for the E1A promoter. The amount at 12 hpi relative to that at 6 hpi was graphed. Mean values with s.d. were obtained from three independent experiments. (C) RT-qPCR assays. Total RNAs were purified at 6 and 12 hpi, and subjected to RT-qPCR using indicated primer sets. The mRNA levels relative to those of control cells at 12 hpi were graphed. Mean values with s.d. were obtained from three independent experiments. *P*-values are calculated using Student's *t*-test. "n.s." indicates "not statistically significant".

siRNA targeted for CTCF (siCTCF) was introduced into HeLa cells, and then cell lysates were prepared and subjected to western blot analyses using anti-CTCF antibody. Only a single band corresponding to CTCF was detected, demonstrating the specificity of the antibody. Under our experimental condition, the expression level of CTCF in siCTCF-treated cells was decreased to approximately 25% of that in control cells (Fig. 1A, compare lane 4 with lanes 1–3).

To test whether CTCF plays a role in Ad DNA replication and gene expression, we carried out CTCF KD followed by quantitative PCR (qPCR) of viral DNA and RT-qPCR (Fig. 1B and C). Under our experimental condition, the onset of viral DNA replication can be observed around 8 hpi (hours post infection)[12,14]. siCont- or siCTCF-treated cells were mock-infected or infected with human adenovirus type 5 (HAdV5) at an MOI (multiplicity of infection) of 100, and at 6 (for early phases) and 12 hpi (for late phases of infection) total DNAs and RNAs were purified. We first measured viral DNA amounts by qPCR using a primer set targeted for the E1A promoter region (E1A pro, see Table 2) to evaluate the efficiency of viral DNA replication (Fig. 1B). In siCont-treated cells, the amount of viral DNA was increased by ~30 fold through viral DNA replication. In contrast, siCTCF-treated cells allowed only ~9 fold amplification of viral DNA.

Next, we performed RT-qPCR assays using several primer sets for cellular and viral genes (Fig. 1C). Under the condition employed here, the mRNA level of *GAPDH* was unaffected by Ad infection and siRNA treatment, and that of *CTCF* was specifically decreased by siCTCF treatment (Fig. 1C, *GAPDH* and *CTCF*). The mRNA levels of viral early genes were not significantly affected by CTCF KD (Fig. 1C, E1A and E4), suggesting that CTCF is not involved in viral early gene expression. In contrast, the level of mRNA transcribed from the major late promoter (MLP) was drastically decreased by siCTCF treatment (Fig. 1C, MLP). Similarly, the mRNA level of E2A was reduced by CTCF KD, particularly at 12 hpi (Fig. 1C, E2A). It is noted that E2 gene transcription is regulated by early and late promoters and transcription from the E2 late promoter depends on viral DNA replication[15] (Unpublished data). Therefore, it is reasonable to assume that CTCF KD could predominantly affect the transcription from the E2 late promoter, although in this study we did not discriminate E2A mRNAs transcribed from two promoters. Collectively, these results suggest that CTCF is critical for viral DNA replication as well as late, but not early, gene expression.

**CTCF binds to viral chromatin in a DNA replication-dependent manner.** To examine whether CTCF functions directly on viral chromatin, ChIP assays were performed using anti-CTCF antibody (Fig. 2). Since the effect of CTCF KD was observed in late phases of infection (Fig. 1), first we studied using infected cells at 12 hpi for ChIP assays (Fig. 2A). We used a variety of primer sets for the Ad genome to test the genome-wide binding of CTCF (see Fig. 2B). We found that CTCF is recruited into several regions of the virus genome, including the MLP region and the ORF regions of the viral structural protein (Hexon) and the E4 ORF3 gene (E4 orf). In addition, a weak binding of CTCF at the E1A pro region was observed. Next, we focused on three CTCF binding sites, the E1A pro, MLP, and Hexon regions, and performed ChIP assays using cells cultured in the absence or presence of hydroxyurea (HU), a DNA replication inhibitor, to examine whether the CTCF binding observed here is DNA replication-dependent (Fig. 2C). The recruitment of CTCF into those regions was observed only at 12 hpi, and this was inhibited by the addition of HU, indicating that CTCF is recruited onto viral chromatin in a DNA replication-dependent manner.

## Discussion

The results obtained in this study indicate that CTCF binds to Ad chromatin depending on its DNA replication and plays a pivotal role in late phases of infection. Our KD experiments clearly reveal that CTCF is required for viral DNA replication and late gene expression (Fig. 1B and C). It is demonstrated that viral late genes are activated depending on its DNA replication[13]. Thus, the mRNA levels of late genes also should be affected when only DNA replication would be directly repressed by CTCF KD. Conversely, viral factors involved in

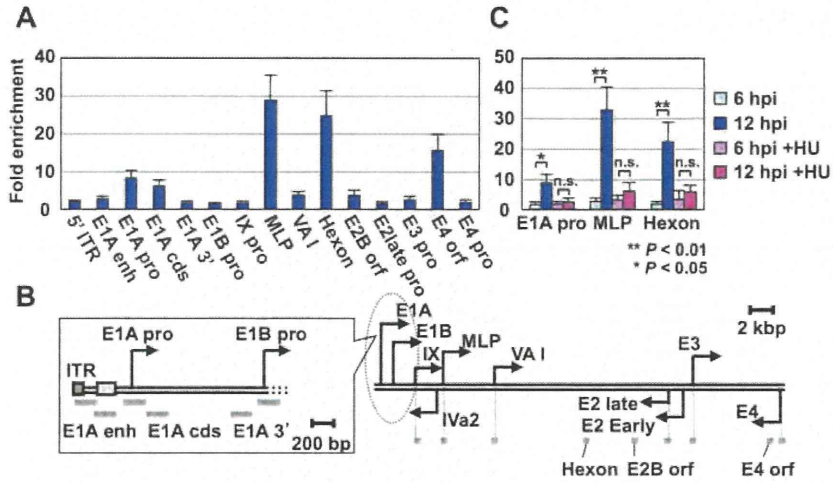


**Figure 2 | CTCF binding on viral chromatin.** (A) ChIP assays with anti-CTCF antibody. HeLa cells were infected with HAdV5 at an MOI of 100, and at 12 hpi subjected to ChIP assays using anti-CTCF and anti-FLAG antibodies. The binding levels were calculated as fold enrichment against that obtained in a negative control (anti-FLAG antibody). Mean values with s.d. were obtained from three independent experiments. (B) Structure of the Ad genome. Arrows represent promoters of viral genes. Target regions for ChIP assays are indicated by gray bars. (C) Effect of viral DNA replication on CTCF binding. HeLa cells were infected with HAdV5 and cultured in the absence or presence of 2 mM HU. At 6 and 12 hpi, ChIP assays were carried out as described above. *P*-values are calculated using Student's *t*-test.

its DNA replication are encoded by the E2 gene, and its expression was suppressed by CTCF KD (Fig. 1C). Therefore, the inhibition of viral late gene expression also could be the cause for less efficient viral DNA replication. Because of this interdependency, we could not precisely discriminate whether CTCF KD primarily affects viral DNA replication or late gene expression (or both). In addition, we could not exclude the possibility that the lower level of the MLP mRNA in siCTCF-treated cells results from less amount of viral DNA templates due to the defect in viral DNA replication. Nonetheless, we speculate that CTCF may be involved in the regulation of late gene expression: First, the binding of CTCF is dependent on viral DNA replication (Fig. 2C), suggesting that this protein possibly functions in the step(s) later than viral DNA replication; second, when the level of the MLP mRNA at 12 hpi was normalized by the amounts of the virus genome, the level of the MLP mRNA per one viral DNA in CTCF KD cells was still lower than that in control cells (Fig. 1, MLP mRNA level: ~15%/virus genome: ~30% = MLP mRNA per one viral DNA: ~50%).

It is an important question how CTCF regulates the function of the virus genome/chromatin. Although in this study we found several CTCF binding sites (Fig. 2A), there could be additional regions for CTCF on the virus genome. Thus, at the moment, it is difficult to dissect a role of each CTCF binding site and the cooperative function among those regions. It is suggested that the E1B, IX, and E2 late genes/promoters are also activated depending on viral DNA replication[15–17] as is the MLP. However, we could not observe the CTCF binding on those regions (Fig. 2A), suggesting that the recruitment of CTCF onto each late promoter seems not to be required for the genome-wide coordination between viral DNA replication and the expression of late genes. Further studies are needed to address these points. As described above, it is shown that Ad DNA replication is required for the activation of viral late genes[13]. Similarly it is reported that DNA replication is essential for the expression of certain cellular genes such as the *HoxB* gene[18], although the molecular details remain to be determined. To our knowledge, this is the first report indicating the possible involvement of CTCF in the DNA replication-dependent activation of the genes. Thus, our findings may provide insight into an uncharacterized mechanism of gene regulation that involves DNA replication.

## Methods

**Cells and viruses.** Maintenance of HeLa cells, and purification and infection of human adenovirus type 5 (HAdV5) were carried out essentially as described previously[12,14]. Hydroxyurea (HU) was added at the final concentration of 2 mM right after infection to block DNA replication.

**Antibodies.** To obtain recombinant CTCF N-terminal region (amino acids (aa) 1–267) as an antigen, the expression vector for His-tagged CTCF(1–267) was constructed. cDNA fragment of full-length CTCF was amplified by PCR with a primer set, 5′-AGGGCATATGGAAGGTGATGCAGTCGAAGCCATTGTGG-3′ and 5′-AGCCTCGAGAAGTCCTGGCGACGCACAAGGCTCCGCC-3′, and cloned into the pBluescript-FLAG vector (pBS-FLAG-CTCF). Using pBS-FLAG-CTCF as a template, cDNA fragment corresponding to aa 1-267 was amplified by PCR with a primer set, 5′-AGGGCATATGGAAGGTGATGCAGTCGAAG-CCATTGTGG-3′ and 5′-GTTGAATTCACTGGAATGTCTTCTTTACAC-3′, and cloned into the pET-14b vector. *E. coli* was transformed with the resultant vector, pET-14b-CTCF(1-267), and His-CTCF(1–267) was expressed and purified using the Ni-NTA resin (Novagen) according to the manufacturer's protocol.

Rabbit anti-CTCF antibody was raised against His-CTCF(1–267) according to standard protocols. Mouse anti-FLAG M2 and mouse anti-β-actin antibodies were described elsewhere[12,14].

**RT-qPCR assays.** RT-PCR and quantitative PCR (qPCR) were performed essentially as described previously[12,14]. Total RNAs were purified by phenol extraction followed by DNase I treatment. cDNA was synthesized from total RNA (1 μg) using ReverTraAce (Toyobo) and oligo-dT primer according to the manufacturer's protocol. qPCR was carried out using FastStart SYBR Green Master (Roche) and

**Table 1 | Primers used for RT-qPCR**

| Primer | Sequence (5′-3′) |
|---|---|
| GAPDH forward | AGCCAAAAGGGTCATCATCTC |
| GAPDH reverse | GGACTGTGGTCATGAGTCCTTC |
| CTCF forward | TGACACAGTCATAGCCCGAAAA |
| CTCF reverse | TGCCTTGCTCAATATAGGAATGC |
| E1A forward | GAGACATATTATCTGCCACGGAG |
| E1A reverse | AGTGAGTAAGTCAATCCCTTCCTG |
| E4 forward | ACAGAACCCTAGTATTCAACCTGC |
| E4 reverse | GACAGCGACATGAACTTAAGTGAG |
| MLP forward | ACTCTCTTCCGCATCGCTGT |
| MLP reverse | GTGACTGGTTAGACGCCTTTCT |
| E2A forward | GTGTAGACACTTAAGCTCGCCTT |
| E2A reverse | CTTCAAACTACTGCCTGACCAAGT |