FULL PAPER

Replication kinetics of turkey herpesvirus in lymphoid organs and feather follicle epithelium in chickens

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ABSTRACT. Marek's disease virus (MDV) is an oncogenic alphaherpesvirus that causes immunosuppression, T-cell lymphomas, and neuropathic disease in infected chickens. To protect chickens from MDV infection, an avirulent live vaccine of turkey herpesvirus (HVT) has been successfully used for chickens worldwide. Similar to MDV for natural infection in both chickens and turkeys, HVT also infects lung in the early stage of infection and then lymphocytes from lymphoid organs. Virus replication requires cell-to-cell contact for spreading and semi-productive lytic replication in T and B cells. Then, cell-free infectious virions matured in the feather follicle epithelium (FFE) are released and spread through the feather from infected turkeys or chickens. To understand the lifecycle of HVT in inoculated chickens via the subcutaneous route, we investigate the replication kinetics and tissue organ tropism of HVT in chickens by a subcutaneous inoculation which is a major route of MDV vaccination. We show that the progeny virus matured in lymphocytes from the thymus, spleen, and lung as early as 2 days post-infection (dpi) and bursa of Fabricius at 4 dpi, whereas viral maturation in the FFE was observed at 6 dpi. Furthermore, semi-quantitative reverse transcription-PCR experiments to measure viral mRNA expression levels revealed that the higher expression levels of the late genes were associated with viral maturation in the FFE. These data that tropism and replication kinetics of HVT could be similar to those of MDV through the intake pathway of natural infection from respiratory tracts.

KEY WORDS: feather follicle epithelium, late gene expression, *Meleagrid herpesvirus*, Turkey herpesvirus, vaccine

Marek's disease (MD) is one of the most important diseases to be controlled, as it causes serious economic losses in the poultry industry [29]. MD is caused by oncogenic virulent Marek's disease virus (MDV), which induces deadly contagious malignant T-cell lymphomas, paralysis, and immunosuppression [20]. Turkey herpesvirus (HVT) belongs to alphaherpesvirus serotype 3 *Meleagrid herpesvirus 1* and has been used as a live vaccine worldwide alone or in combination with other serotypes to prevent MD caused by the virulent MDV [25, 28].

HVT naturally infects turkeys; however, it is apathogenic and non-oncogenic against chickens. The HVT-based vaccine, which successfully confers immunity against MDV infection, has been in use since the early 1970s. Nonpathogenic SB-1 strain (serotype 2) and attenuated CVI988 Rispens strain (serotype 1) vaccines have been also used since the 1980s and the 1990s, respectively. The HVT vaccine has been utilized in combination with the CVI988 Rispens strain or SB-1 strain as an effective bivalent vaccine to counter more virulent MDV strains [26, 28]. Previous studies revealed that the combination of SB-1 and HVT vaccination synergistically prevented lymphoma in chicks [27, 30] and mutually induced several cytokines and interferon-stimulating genes, indicating that the combination with HVT vaccine enhanced cellular immunity against MDV infection in chickens [23]. Although the HVT vaccine is generally inoculated to day-old chicks subcutaneously or *in ovo* to confer protection from MDV infection, it remains unelucidated how and when the HVT vaccine replicates and stimulates immune responses in vaccinated chickens via these routes to establish protection against virulent MDVs.

The life cycle of MDV for replication in chickens is initiated by the inhalation of cell-free virus particles in dander or dust from chicken skin and/or feather follicle epithelium (FFE) [1, 5]. Following the semi-productive lytic infection in the B and T cells in each lymphoid tissue, the infected CD4+ cells also transfer the virus to the FFE, and the virus undergoes fully productive replication in the epithelium [2, 3]. Finally, the infectious virus particles are released from the FFE into the environment through dust containing the feather dander of infected chickens and horizontally spread to contact chickens in the same poultry house. Unlike MDV in chickens, previous reports showed that efficient horizontal transmission of HVT via airborne route between

chickens was not occurred because of poor shedding of infectious HVT from the HVT-infected chickens [6]. Although the HVT protein was detected in lymphoid organs and FFE in chickens inoculated via intraperitoneal [11], the accurate tropism of tissues and organs and the replication kinetics of HVT in subcutaneously vaccinated chickens remained to be elucidated.

In this study, to further understand the spatiotemporal dynamics of HVT subcutaneously administered for MDV infection, we focused on the overall replication kinetics of HVT in chickens after administration via a subcutaneous route, but not a natural (oral) route. We also investigated the viral genome replication and the maturation of the virus in each lymphoid organ and FFE in a time course study. Comparison of the replication kinetics and tropism for HVT with those for MDV in chickens is helpful information to maximize the potential vaccine efficacy of HVT against the virulent MDV by the optimized vaccination routes.

MATERIALS AND METHODS

Cells and virus

Specific pathogen-free (SPF) embryonated eggs of White Leghorn at 10-day old were subjected to the preparation of chicken embryonated (CE) cells. The CE cells were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5% fetal bovine serum (Cytiva, Marlborough, MA, USA), 20 U/ml Penicillin G potassium (Meiji Seika Pharma, Tokyo, Japan), 100 µg/ml Streptomycin sulfate (Meiji Seika Pharma, Tokyo, Japan), and 250 µg/ml amphotericin B (Wako, Osaka, Japan). The CE cells were seeded into 6-well plates (3×10^6 cells/well). HVT FC126 strain was isolated from turkeys [31] and was provided from Maine Biological Laboratories (Salisbury Cove, ME, USA) and propagated in the CE cells for the assays.

Sample collection and isolation of cell-free virion

SPF White Leghorn chickens were purchased from VALO Biomedia (Osterholz-Scharmbeck, Germany) and housed in isolation units in our laboratory. All animal experiment procedures were conducted in accordance with relevant national and international guidelines defined in our laboratory for the humane use and care of chickens. One day-old chicken was subcutaneously inoculated at neck with a dose of 1.7×10^5 focus-forming unit of virus to accurately clarify the time of the virus isolation and viral DNA detection from each organ at an earlier stage of infection from 1 dpi. Their bursa of Fabricius, thymus, spleen, and lung or feathers from five chickens were collected at 1, 2, 3, 4, and 7 dpi or 1–7, and 28 dpi. The five lymphoid organs and lungs were pooled and minced with scissors to release lymphocytes into the supernatant, and the cells were suspended in Eagle's minimum essential medium supplemented with 5% fetal bovine serum and 10% dimethyl sulfoxide for storage at −70°C until use. Feathers from the five chickens were pulled out, and the FFE was obtained from the feather sheath. The FFE was also minced with scissors and briefly sonicated for 1 min at 20 kHz on ice in sucrose-phosphate-glutamine-albumin (SPGA) buffer to release the virus from the FFE into the supernatant, resulting in FFE extract containing the virus.

Quantification of the amount of viral DNA using duplex qPCR

Viral DNAs from the isolated lymphocytes or virus extracted from the FFE were extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified viral DNAs were subjected to a duplex qPCR using specific primers for HVT SORF1 and chicken α2 (VI) collagen genes (Table 1), as described previously [12]. The qPCR was carried out using a StepOne real-time system (Applied Biosystems, Foster City, CA, USA) with TaqMan probes according to the manufacturer's protocols. The ΔCT values of the viral DNAs from lymphocytes in each organ or the FFE extract were calculated with each CT value of the chicken α2 (VI) collagen gene as an internal control, and the relative expression levels of the viral DNAs were further calculated using the comparative CT method ($2^{-\Delta\Delta Ct}$ method).

Passage of viruses in CE cells and indirect immunofluorescence

CE cells were infected with viruses from the FFE, and the cells were incubated for 7 days at 37°C. At 7 dpi, the cells were dissociated with 0.05% trypsin and 100 µl of the dissociated cells were passaged to the new CE cells. These procedures were repeated twice, and cytopathic effects (CPE) were observed. Micrographs were taken using an inverted microscope (CKX53; Olympus, Tokyo, Japan). In the final passage (3rd passage), the cells were grown on coverslips and fixed in 3.7% formaldehyde. After blocking with 3% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), the cells were incubated with a primary chicken anti-HVT serum obtained from chickens vaccinated with the HVT FC126 strain and subsequently with a rabbit anti-chicken IgY secondary polyclonal antibody conjugated with FITC (Bethyl Laboratories, Montgomery, TX, USA). The coverslips were mounted on glass slides using mounting medium (10% glycerol in PBS). The cells were then observed with a fluorescent microscope (ECLIPSE Ts'2R; Nikon, Tokyo, Japan).

Semi-quantification of viral mRNA expression levels

To obtain viral mRNA from FFE samples at 5, 6, and 7 dpi or from the samples of the thymus at 1 and 2 dpi, the total RNA was purified, and contaminating DNA was removed using NucleoSpin RNA (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's instructions. Approximately 1.5 µg of total RNA was used for the amplification of viral mRNA via reverse transcription (RT)-PCR using QIAGEN OneStep RT-PCR Kit (Qiagen). The one-step RT-PCR was performed in a 10-µl reaction mix, and each mRNA was amplified through a series of processes including denaturation, annealing, and extension by cycle numbers (21, 25, 27, 29, 33, and 36) to semi-quantify the mRNA expression levels using the HVT gene-specific or GAPDH

Table 1. Oligonucleotide sequences used for qPCR for the SORF1 gene of herpesvirus (HVT) and chicken α2 (VI)-collagen gene

Molecules	Primers (5′ to 3′)
HVT-SORF1	GGCAGACACCGCGTTGTAT
	TGTCCACGCTCGAGACTATCC
	Probe: FAM- AACCCGGGCTGACGTCTTC-BHQ1
Chicken α2 (VI)-collagen	GGGAACTGGAGAACCCAATTTT
	CGTGCCGCTGTCTCTACCAT
	Probe: FAM- CCCTTAACTGCTACTGCAG-BHQ1

Table 2. Oligonucleotide sequences used for semi-qRT-PCR for HVT080, HVT085, HVT012, HVT060, HVT052, HVT053, and chicken GAPDH genes

Molecules	Primers (5′ to 3′)
HVT080	ATGGCATCCCCTTCAGAATATGACC
	TGGAGAAGGGACGGTCTACTGAAGA
HVT085	ATGGATGGGAAAACTACACACGTGA
	ACCTAGCATATACATATCAAACAAA
HVT012	ATGGCTCGTAGTTCAACTGACAGGTT
	TGTAGTGCCAGTGATTATACAGTCC
HVT060	ATGATGGATCAAAGCCTTAAGTCTG
	GGCAGAATTCAATTCTTAGACTCTC
HVT052	ATGGTTTCCAACATGCGCGTTCTAC
	TATATCAGGTATTTTGTGAGAAGAG
HVT053	ATGATGTCGCCCACCCCTGAAGATG
	TTGCATTTTTCCCGGCCACGCGCAT
Chicken GAPDH	GGTGGTGCTAAGCGTGTTAT
	ACCTCTGTCATCTCTCCACA

primers (Table 2). After the RT-PCR, the PCR products were analyzed on 1% agarose gel, followed by staining with ethidium bromide. The band intensity of each viral mRNA was normalized to that of GAPDH using ImageJ software. Statistical significance was determined using the Student's *t* test. *P* values <0.05 or 0.01 were considered statistically significant.

RESULTS

Virus isolation correlated with amounts of HVT DNA from lymphocytes in lymphoid organs and lungs during early infection but not with that from the FFE

To understand the replication kinetics of HVT in chickens, we subcutaneously administered one-day-old chickens with HVT in the neck and collected central and peripheral lymphoid organs such as the bursa of Fabricius, thymus, and spleen to isolate infected lymphocytes such as T cells and B cells at various time points. The lung was also collected from infected chickens to examine whether the infected lymphocytes were isolated from the lung. The FFE is the only tissue in which fully infectious viral particles are produced and released. The epithelium was extracted by scraping the tissue from the feather sheath of infected chickens. Our qPCR experiment showed that HVT DNA was present in all the harvested organs and FFE at 1 dpi, although the viral DNA levels were low and virus isolation was unsuccessful (Fig. 1A and 1B). However, the viral DNA dramatically increased in a time-dependent manner, and cell-associated viruses were first isolated at 2 dpi from the thymus, spleen, and lung but not from the bursa of Fabricius, possibly due to the low amount of viral DNA in the bursa. The relative levels of viral DNA gradually increased day by day, and cell-associated viruses from the bursa were first detected at 4 dpi. Interestingly, the viruses were first isolated from the FFE at 6 dpi but not at 5 dpi, even though a comparable amount of viral DNA in the FFE at 5 dpi to that at 6 dpi was detected (Fig. 1A and 1B). The viral DNA was still detected at 28 dpi from the FFE; however, the virus was not isolated (data not shown), consistent with a previous study [11]. To confirm virus maturation in the FFE at 5 dpi, HVT protein expression in CE cells was examined by indirect immunofluorescence using anti-HVT serum after coculture with FFE extract. When the FFE extract was inoculated to the CE cells, no CPE and viral protein expression was observed in the cells. The CE cells were then passaged twice to new CE cells to propagate the virus. As expected, even after two passages in CE cells, no fluorescent signal or CPE was observed in the passaged cells. On the other hand, the CE cells cocultured with FFE extract at 6 and 7 dpi showed CPE and fluorescent signals (Fig. 2A and 2B). The qPCR experiment showed that the virus DNAs extracted from the FFE at 5 dpi were detectable (Fig. 1A). However, the viral DNA level was below detection limit after two passages in CE cells cocultured with FFE extract at 5 dpi (Fig. 2C), indicating no viral maturation in the FFE at 5 dpi but not at 6 and 7 dpi. Taken together, these data suggest that HVT DNA levels are not linked to viral isolation from the FFE.

High-level transcription of late genes in the FFE is involved in viral maturation

Herpesviruses exhibit three distinct phases tightly regulated by a temporal cascade for viral transcription: immediate-early, early, and late genes [9, 13, 14, 24]. Late genes mainly encode the viral structural proteins that are required for the formation of infectious viruses, such as viral assembly and release [17, 21]. A transition of gene expression phases from early to late gene expressions is dependent on the onset of viral DNA replication with maximal expression level [10, 15, 32]. To examine whether a high transcription level for late genes in the FFE is involved in virus maturation, we carried out semi-qRT-PCR to analyze the expression levels of the viral mRNA in the FFE at 5, 6, and 7 dpi. For each phase of gene expression, the following HVT genes were chosen for semi-qRT-PCR as representatives: immediate-early genes, HVT080 (ICP4) and HVT085 (ICP22); early genes,
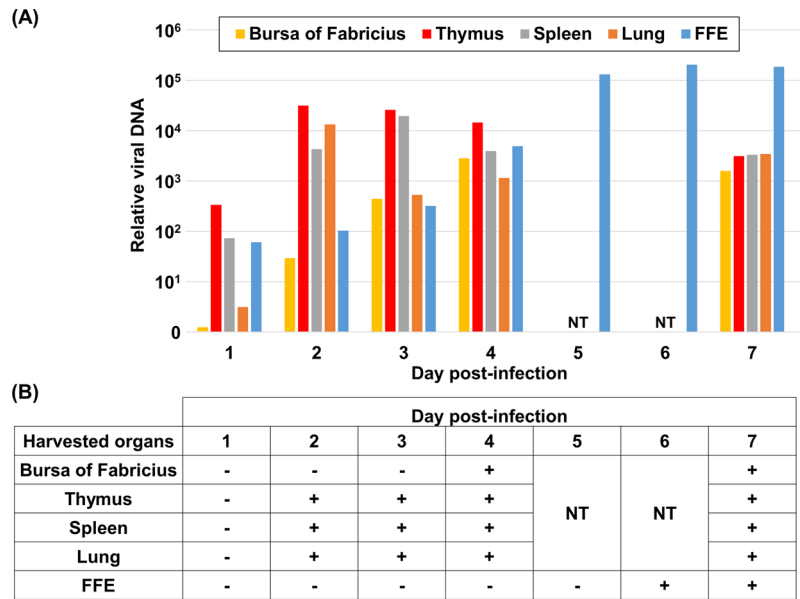
(B)

Harvested organs	Day post-infection						
	1	2	3	4	5	6	7
Bursa of Fabricius	-	-	-	+	NT	NT	+
Thymus	-	+	+	+	NT	NT	+
Spleen	-	+	+	+	NT	NT	+
Lung	-	+	+	+	NT	NT	+
FFE	-	-	-	-	-	+	+

Fig. 1. Temporal study of Turkey herpesvirus (HVT) tissue tropism in chickens infected with HVT and cytopathic effect (CPE) by coculturing chicken embryonated (CE) cells with lymphocytes derived from HVT-infected bursa of Fabricius, thymus, spleen, lung, or feather follicle epithelium (FFE) extracts. **(A)** One-day-old chickens were subcutaneously administered with HVT in the neck. At various time points (indicated), each organ or feather from infected chickens was collected and lymphocytes or FFE extract was obtained, respectively. Viral DNA was extracted and subjected to a duplex quantitative PCR using gene-specific primers for the SORF1 gene and chicken $\alpha2$ (VI)-collagen gene for internal control. The relative expression levels of the viral DNA were calculated using the comparative CT method ($\Delta\Delta$CT method). **(B)** The obtained lymphocytes or FFE extract was cocultured with CE cells to detect mature viruses by CPE appearance. The CE cells were incubated for 7 days and passaged to the next new CE cells twice, followed by further incubation to observe whether CPE appeared or not. +, CPE positive. -, CPE negative. NT, Not Tested.
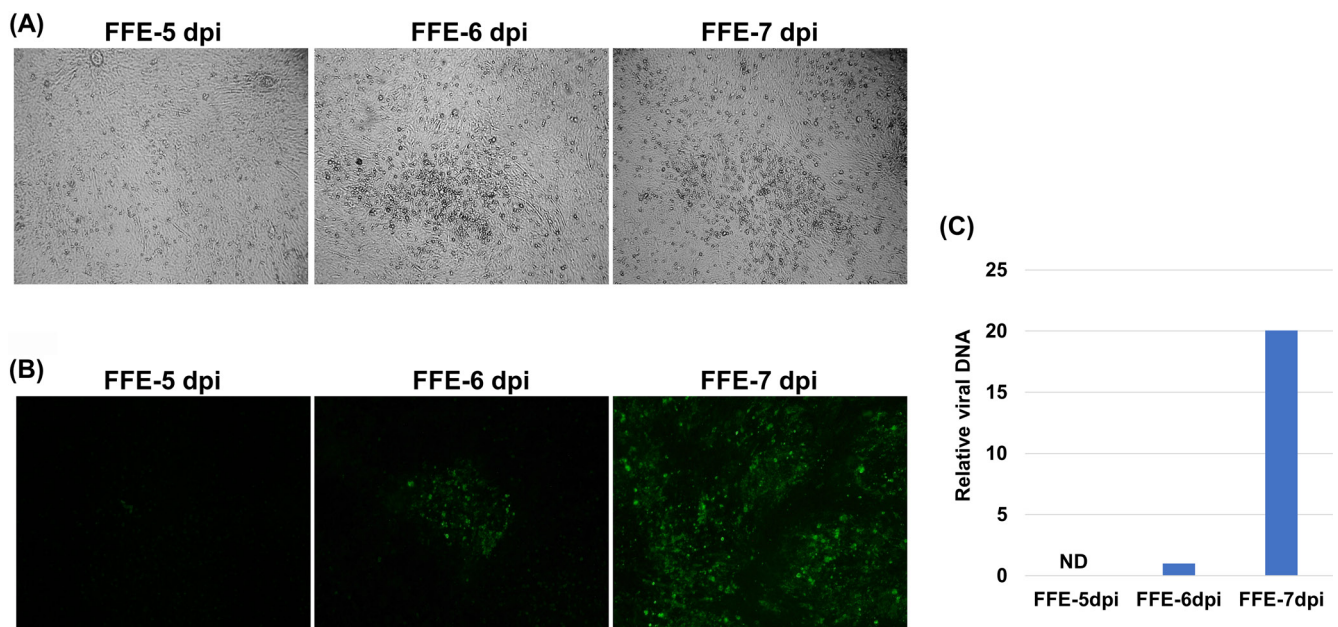

Fig. 2. No mature virus in the feather follicle epithelium (FFE) at 5 dpi. Chicken embryonated cells were inoculated with viruses isolated from the FFE at 5, 6, and 7 dpi, and the cells were incubated for 7 days, followed by passaging three times. After the 3rd passage, the cells were observed via inverted microscopy with a 10× objective lens **(A)**. For immunofluorescence assay, the cells were fixed and incubated with α-HVT serum (green signals) to stain the HVT viral proteins. The stained cells were observed with an immunofluorescence microscope **(B)**. For qPCR, viral DNAs were extracted from the cells after the 3rd passage and subjected to the qPCR using the gene-specific primers for SORF1 gene of HVT and chicken $\alpha2$ (VI)-collagen gene for internal control. The relative expression levels of viral DNA were calculated as described in the legend to Fig. 1A. No HVT DNA was detected in the FFE at 5 dpi after passaging twice.

HVT012 (UL5) and HVT060 (UL52); and late genes, HVT052 (UL44) and HVT053 (UL45). The semi-qRT-PCR analysis revealed that the transcription levels for both HVT052 and HVT053 late genes were significantly upregulated in the FFE at 7 dpi compared with those at 5 dpi, whereas no significant differences in the expression levels of immediate-early and early genes were observed among the FFE at 5, 6, and 7 dpi, except for the expression level of the HVT085 gene between 5 and 6 dpi (Fig. 3A and 3B). When the HVT DNA in the FFE at 5 dpi was quantified by qPCR, the DNA level almost reached a peak of viral DNA replication (Fig. 1A), leading to the onset of viral DNA replication in the FFE at 5 dpi and the shift to the late gene expression phase for the formation of mature viruses. Similarly, the level of each viral mRNA of the infected lymphocyte cells from the thymus at 1 and 2 dpi was examined. The mRNAs of immediate-early and early genes were detected in the samples of the thymus, even at 1 dpi. In contrast, no or very faint bands of late genes were detected in the thymic samples at 1 dpi (Fig. 4A and 4B). Subsequently, the expressions of the immediate-early and early genes significantly increased at 2 dpi. Interestingly, even though viruses were first isolated at 2 dpi, late gene expressions did not dramatically increase, especially HVT052 gene expression, compared with those of immediate-early and early genes (Fig. 4A and 4B), suggesting that the pattern of late viral gene expression in the thymus and FFE was not consistent.
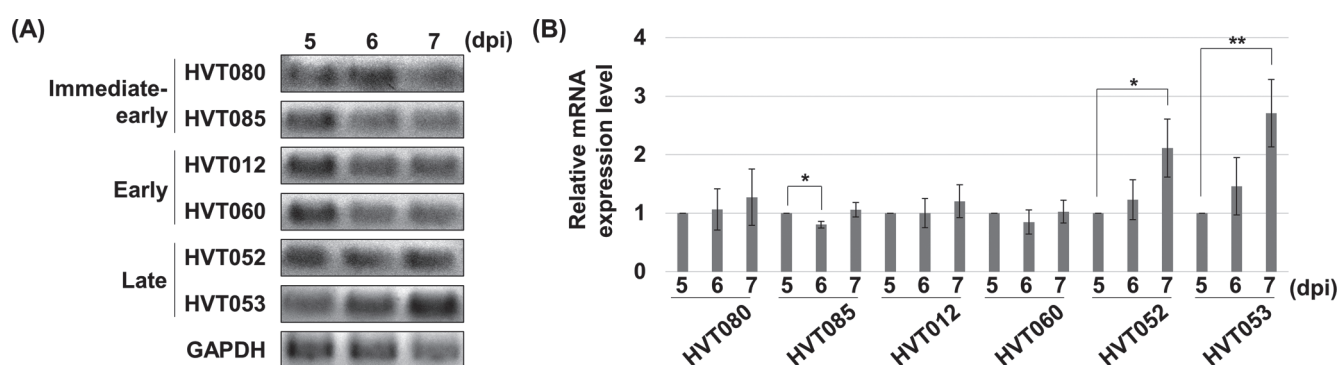

Fig. 3. A high-level expression of Turkey herpesvirus (HVT) late gene in the feather follicle epithelium (FFE) at 6 and 7 dpi. Each total RNA content from FFE extract at 5, 6, and 7 dpi was purified and subjected to the semi-qRT-PCR to semi-quantify the mRNA expression levels for HVT080, HVT085, HVT012, HVT060, HVT052, and HVT053 using HVT gene-specific primer sets at the end of 29 cycles for the FFE **(A)**. Intensities of signals were measured using ImageJ software and relative intensities (6 and 7 dpi to 5 dpi for the FFE) were calculated **(B)**. Data are shown as means with standard deviations from three independent experiments. Asterisks represent statistically significant differences (*$P<0.05$; **$P<0.01$) by Student's *t*-test.
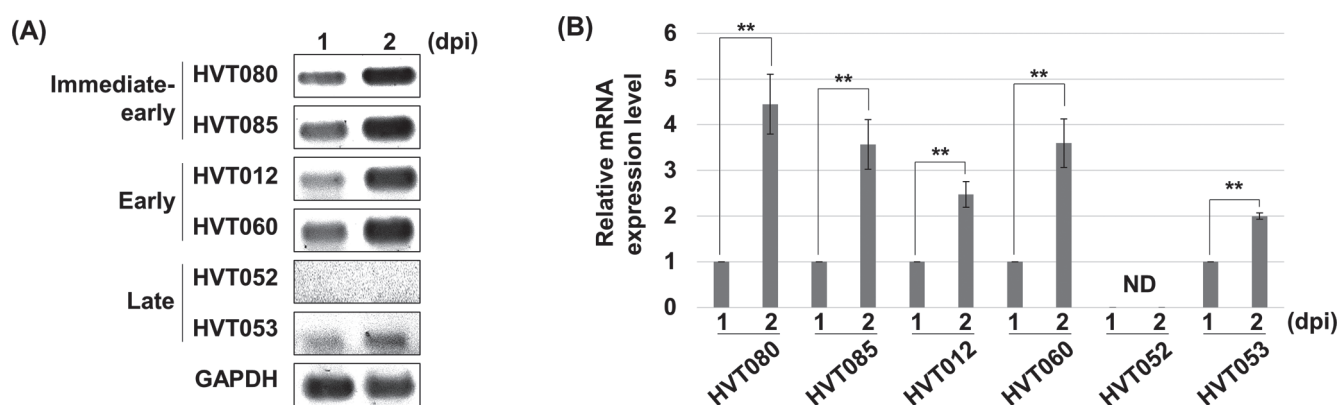

Fig. 4. A low-level expression of Turkey herpesvirus (HVT) late gene in the thymus at 2 dpi. Each total RNA content from the thymus at 1 and 2 dpi was purified and subjected to the semi-qRT-PCR to semi-quantify mRNA expression levels as described in the legend of Fig. 3A at the end of 36 cycles **(A)**. Intensities of signals were measured using ImageJ software and relative intensities (1 to 2 dpi for the thymus) were calculated **(B)**. Data are shown as means with standard deviations from three independent experiments. Asterisks represent statistically significant differences (**$P<0.01$) by Student's *t*-test. ND, Not Detected.

DISCUSSION

Vaccination is a very effective way of protecting chickens from immunosuppression and tumorigenesis caused by virulent MDVs. Investigating the replication kinetics of HVT in chickens is important to understand the lifecycle of HVT in vaccinated chickens. Nevertheless, less information is available regarding the detailed analysis of replication kinetics for HVT in chickens. A previous study on replication kinetics for the MDV CVI988 vaccine strain in one-day-old chickens showed that virus isolation and viral DNA detection by qPCR were first confirmed at 5 and 10 dpi from the spleen and feather, respectively. However, viral DNA detection and virus isolation in an early period for the spleen and feather were not examined in this report. The qPCR was carried out only to detect viral DNA for the samples from the thymus and bursa of Fabricius at 0–4 dpi [2]. Therefore, it is unclear whether the increase in the amount of viral DNA is directly correlated with viral maturation for the isolation of the virus from lymphocytes and the FFE. Furthermore, a little detailed analysis of the replication kinetics and tissue tropism of HVT in chickens at an earlier time after inoculation has been reported to date [11, 27].

In this study, we examined the replication kinetics of HVT in various lymphoid organs, lungs, and the FFE by virus isolation and monitoring the level of viral DNA expression. In the preliminary tests, we first administered one dose of HVT (approximately 3000 PFU/dose) to one-day-old chickens and obtained the infected lymphocytes from the thymus, spleen, bursa, and FFE. Although the viral DNA levels from each organ and FFE were very low, the replication kinetics and the tropism were similar to those obtained from the chickens administered with the 1.7×10^5 FFU of HVT (data not shown). To obtain more reliable results and increase test sensitivity, we administered the 1.7×10^5 FFU of HVT to one-day-old chickens. After administration of the HVT, we harvested each organ and FFE from the chickens in a time-dependent manner and then attempted to extract the cell-free matured virus particles from lymphocytes in the thymus and FFE by sonication. Although a very small amount of the matured virus from lymphocytes in the thymus was obtained, the viral maturation period of cell-free virus in lymphocytes was consistent with that of cell-associated virus in lymphocytes. We also failed to obtain the cell-associated virus from FFE by co-culturing with CE cells. Therefore, we investigated the viral maturation periods in lymphocytes and FFE by obtaining the cell-associated and cell-free viruses, respectively. Inconsistent with a previous report showing that the replication of HVT initiated high in the bursa at 1 dpi via intraperitoneal inoculation [27], the expression level of HVT DNA was lower in the early phase of infection in the bursa of Fabricius compared with that in other lymphoid organs, and it gradually increased in a time-dependent manner (Fig. 1A). Viral isolation was then delayed at 4 dpi (Fig. 1B). A previous study comparing the inoculation routes via intratracheal with via intraperitoneal reported the differences of viremia phenotype, tumorigenesis, and replication kinetics of MDV in chickens, presumably due to different host immunity responses by the inoculation routes regarding viral replication in organs [4]. Therefore, it is possible that the difference of the inoculation routes through the intraperitoneal in the previous study [27] and subcutaneous in this study affect the kinetics of HVT replication in the bursa.

The cellular immunity by activated T cells and secreted cytokines at local infected organs is important to protect the chickens from MDV infection [23, 27]. Previous studies showed that higher levels of interferon-γ were induced from splenocytes infected with HVT and SB-1 than those of only HVT [23] and the bivalent vaccination of HVT+SB-1 showed a higher protection rate due to the inhibition of viral replication of a challenge strain in lymphoid organs than that of only HVT [27]. Although it could be difficult to make a direct comparison of the tropism for HVT in chickens following the subcutaneous injection with that for MDV via intraoral route as natural infection, the similarities in the previous study that showed MDV tropism in chickens following intratracheal inoculation [4] were found in this study showing the tropism to at least spleen and lung for HVT even via the subcutaneous route (Fig. 1A). It is conceivable that the HVT tropism similar to the MDV to the local lymphoid organs or lungs could be important as a vaccine efficacy to inhibit the viral replication of the virulent MDV strain.

In addition to MDV, cell-free HVT is also released into the environment via infected, dander, or contaminated dust [8]. To spread many virus particles to the environment, cell-free viruses are produced only in the FFE of infected chickens where the highest viral antigen expression of MDV is seen compared with other tissues [2, 7]. Consistent with these reports, we found that very high levels of HVT DNA were detected by qPCR from FFE extract at 5–7 dpi (Fig. 1A). However, mature viruses were not isolated from the FFE at 5 dpi, even though the level of HVT DNA was similar to that at 6 and 7 dpi (Fig. 1B). In general, herpesviruses exhibit three distinct phases for viral transcription tightly regulated by a temporal cascade: immediate-early, early, and late genes [9]. The first phase of kinetics initiates the gene expression of the immediate-early genes associated with the regulation of transcription for early genes [13, 14]. The early-gene proteins function for viral DNA genome replication and are responsible for the gene expression of late genes [24]. Late genes mainly encode viral structural proteins, which are required for the formation of infectious viruses, such as viral assembly and release [17, 21, 22]. A transition of phases from early to late gene expressions is dependent on the onset of viral DNA replication with maximal expression level [10, 15, 21]. When DNA replication is inhibited with phosphonoacetic acid, late gene expression is also severely inhibited [18, 19], indicating that late gene expression is dependent on continuous viral DNA replication. Our qPCR experiment to quantify the viral DNA in FFE extract revealed that the viral DNA level at 5 dpi almost reached a peak and slightly continued to increase by 7 dpi (Fig. 1A), indicating the onset of viral DNA replication at this time point and possible initiation of the expression of late genes. A previous study has shown that the late gene UL47 was expressed at low levels and predominantly localized in the nucleus in lymphocytes from the spleen, whereas UL47 was highly expressed and mainly localized in the cytoplasm of the FFE, suggesting upregulation of late genes in the FFE [16]. Consistent with this report, our semi-qRT-PCR experiment showed that the expression of late genes HVT052 (UL44) and HVT053 (UL45) in the FFE dramatically increased, and mature viruses were successfully isolated at 6 and 7 dpi (Figs. 1A and 3A), leading to the formation of mature viruses by the high expression of late genes. In contrast, high expressions of late genes

at 6 and 7 dpi in the FFE were significantly unaffected by the levels of immediate-early and early gene expressions. Interestingly, unlike the gene expression in the FFE, the expression levels of late genes, especially HVT052, in the thymus at 2 dpi were lower than those in the FFE at 7 dpi (Figs. 3A and 4A), even though viruses were isolated at 2 dpi in the thymus (Fig. 1A). Therefore, it is conceivable that viral maturation in lymphocytes and the FFE are strictly and differently regulated by gene expression patterns, levels, and combinations, possibly facilitating the generation of the virus in the FFE by the high-level expression of late genes. Further experiments could be required to examine how late gene expressions are important for the maturation of the virus in lymphocytes of the thymus or other organs, and the possibility that other viral or host factors could be involved in viral maturation cannot be ruled out. Furthermore, it remains unelucidated how gene expression regulates and controls cell-to-cell infection between lymphocytes and viral maturation in the FFE to spread infection among chickens.

CONFLICT OF INTEREST. The authors have nothing to disclose.

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