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The large-scale production of an artificial influenza virus-like particle vaccine in silkworm pupae

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ABSTRACT

We successfully established a mass production system for an influenza virus-like particle (VLP) vaccine using a synthetic H5 hemagglutinin (HA) gene codon-optimized for the silkworm. A recombinant baculovirus containing the synthetic gene was inoculated into silkworm pupae. Four days after inoculation, the hemagglutination titer in homogenates from infected pupae reached a mean value of 0.8 million hemagglutination units (HAU), approximately 2,000 μg HA protein per pupa, more than 50-fold higher than that produced with an embryonated chicken egg. VLPs ranging from 30 nm to 300 nm in diameter and covered with a large number of spikes were detected in the homogenates. The spikes were approximately 14 nm long, similar to an authentic influenza HA spike. Detailed electron micrographs indicated that the VLP spike density was similar to that of authentic influenza virus particles. The results clearly show that the expression of a single HA gene can efficiently produce VLPs in silkworm pupae. When chickens were immunized with the pupae homogenate, the hemagglutination inhibition titer in their sera reached values of 2,048–8,192 after approximately 1 month. This is the first report demonstrating that a large amount of VLP vaccine could be produced by single synthetic HA gene in silkworm pupae. Our system might be useful for future vaccine development against other viral diseases.

1. Introduction

In recent years, occasional outbreaks of highly pathogenic avian influenza (HPAI) viruses caused by the H5 and H7 subtypes have occurred in various regions of the world and they have killed a tremendous number of chickens [1–3]. The 1997 H5N1 outbreak in Hong Kong was the first case in which not only chickens but also 18 humans were infected, 6 of whom died [3–5]. Since then, outbreaks of the virus have occurred worldwide, and the threat of H5N1 infection remains.

Because the World Health Organization has highlighted the risk of a human pandemic resulting from the HPAI virus [6], avian influenza vaccines for chickens and humans have been developed or are currently under development [7,8].

Although studies to create more effective and safer vaccines are ongoing worldwide [7–11], the development of a vaccine against HPAI H5 viruses is associated with intrinsic problems such as the low immunogenicity of the virus [12] and the biohazard risk of using an infectious HPAI virus for the vaccine production. To overcome these hurdles, we synthesized an H5HA gene that lacks 4 basic amino acids associated with the pathogenicity of HPAI viruses located between the HA1 and HA2 subunits. The synthesized gene was codon-optimized for the silkworm. We then produced a recombinant baculovirus containing the synthetic HA gene and used it to inoculate silkworm pupae for mass production of the HA protein.

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Using this method, we produced large amounts of the HA protein present in influenza virus-like particles (VLPs).

2. Materials and methods

2.1. Evolutionary analysis

The sequence data used for the analysis are shown in Supplemental Tables 1–5. The synonymous substitution distances were estimated using the Nei and Gojobori method [13]. Phylogenetic trees were constructed using the neighbor-joining method [13,14].

2.2. Cells and viruses

Silkworm Bm-N cells and MDCK cells were maintained in TC-100 medium and minimal essential medium containing 10% fetal bovine serum, respectively. The P6E strain of the *Bombyx mori* nuclear polyhedrosis virus (BmNPV)[15] was used to generate the H5HA-BmNPV recombinant virus. Baculoviruses were propagated in Bm-N cells. The avian influenza viruses used for the hemagglutination inhibition (HI) test (shown in Fig. 4D) were propagated in 10-day-old embryonated chicken eggs (37 °C, 2 days).

2.3. Generation of H5HA-BmNPV

Synthesis of the H5HA gene was carried out by Dragon Genomics (Shiga, Japan). The synthesized gene was inserted into the pBM-8 plasmid [15] using the In-Fusion technique (Clontech, Mountain View, CA, USA) to produce pBM-8-H5HA. Next, pBM-8-H5HA and linearized BmNPV genomic DNA were co-transfected into Bm-N cells, which were then cultured in TC-100 medium without serum. After 5 h, the medium was changed to TC-100 containing 10% fetal bovine serum and the cells were further cultured for 5–7 days. The medium was then harvested and centrifuged at 3000 rpm for 4 min to remove cell debris. The resulting supernatant was used as recombinant virus H5HA-BmNPV.

2.4. Antibodies and Western blot analysis

The anti-FLAG mouse monoclonal antibody M2 and the anti-DYKDDDDK-tag mouse monoclonal antibody were purchased from Sigma (St. Louis, MO, USA) and Wako (Osaka, Japan), respectively. The fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody and the peroxidase-conjugated goat anti-mouse IgG antibody were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). For the Western blot analysis, the peroxidase-conjugated IgG antibody was visualized using an ECL detection system (GE Healthcare, Port Washington, NY, USA), following the manufacturer's instructions.

2.5. Hemadsorption test

Bm-N cells infected with viruses were washed 3 times with phosphate-buffered saline (PBS) and then overlaid with 0.5% (v/v) chicken erythrocytes in PBS. After 30 min, the cells were washed thoroughly with PBS and observed under a microscope.

2.6. Hemagglutination test and hemagglutination inhibition tests

Hemagglutination (HA) and HI tests were performed as described previously [16].

2.7. Production of HA protein in pupae

Silkworm pupae were injected with 100 μL of H5HA-BmNPV in TC-100 (Fig. 4A). Four days later, the infected pupae were homogenized in PBS containing 0.01% formalin and phenylthiourea as an antioxidant; homogenization was performed using a Handy Sonic (Model Tomy UR-20P). Sonication was performed on ice by using 6 pulses, each lasting 2 min. The resultant homogenate was then centrifuged at 6,000 rpm for 30 min. The supernatant was further centrifuged at 16,000 rpm for 30 min. The supernatant from the second centrifugation was used for subsequent experiments.

2.8. Sucrose density gradient centrifugation and electron microscopy

The homogenate of H5HA-BmNPV-infected pupae was centrifuged through a 10–50% (w/w) sucrose density gradient at 25,000 rpm for 120 min using an SW28 swing rotor (Hitachi). The gradient was fractionated and the fractions were examined for HA activity and protein concentration. Six fractions (21–26) that had high HA activity (greater than 8,192) were collected and centrifuged at 25,000 rpm for 4 hr. The pellet was examined under an H-7600 electron microscope (Hitachi) after staining with 2% phosphotungstic acid.

3. Results

3.1. Selection of the target strain used for the anti-H5N1 influenza vaccine

We selected a vaccine strain based on an evolutionary analysis of H5HA genes. Through precise evolutionary analyses of genotype Z viruses, which appeared in 2002 and became the predominant genotype associated with HPAI outbreak in domestic poultry [17], we found that viruses from subclade 2.3, which included the A/tufted duck/Fukushima/16/2011 (H5N1: duck-Fukushima) strain, show discontinuous evolution (i.e., evolution that did not occur at a single rate over time) at the slow rates of 0.002 substitutions/site and 0.008 substitutions/site per year (subclades 2.3 (i) and (ii) in Fig. 1B, respectively). Considering the slow evolutionary rate of wildfowl influenza viruses (0.005 substitutions/site per year, as shown in Fig. 1A), we hypothesized that fast continuous linear evolution would occur in domestic poultry (Fig. 1B red line) and the slow rate of subclade 2.3 would occur in wildfowl. The atypical evolution of subclade 2.3 may be explained if subclade 2.3 viruses are maintained in poultry (Fig. 1B Clade 2.3, red line) but occasionally infect wildfowl, in which they would then evolve at a lower evolutionary rate. If the hypothesis were correct, subclade 2.3 viruses should be the major virus type disseminated; therefore, we selected the duck-Fukushima strain as the target for vaccine preparation.

3.2. Design of the synthetic H5HA gene and the generation of H5HA-BmNPV

The synthetic gene for the production of the H5HA protein in silkworms was designed based on the nucleotide sequence of the HA gene from the duck-Fukushima strain. Duck-Fukushima is a virulent strain that is highly pathogenic to chickens. The polybasic sequence of RERRRKRG, located at the end of the HA1 subunit, is associated with the high pathogenicity of H5N1. Because this sequence is present, the HA0 precursor is cleaved to HA1 and HA2 in almost any cell in which the HA protein is produced. By cleavage, the HA protein obtains membrane fusion activity and is destabilized in acidic environments such as in intracellular transport vesicles [18,19]. Therefore, the RERRRKRG sequence was replaced with RDTRG to avoid this cleavage in insect cells (Fig. 2). By the introduction of RDTRG sequences instead of RERRRKRG, virulent DNA was converted to avirulent DNA.

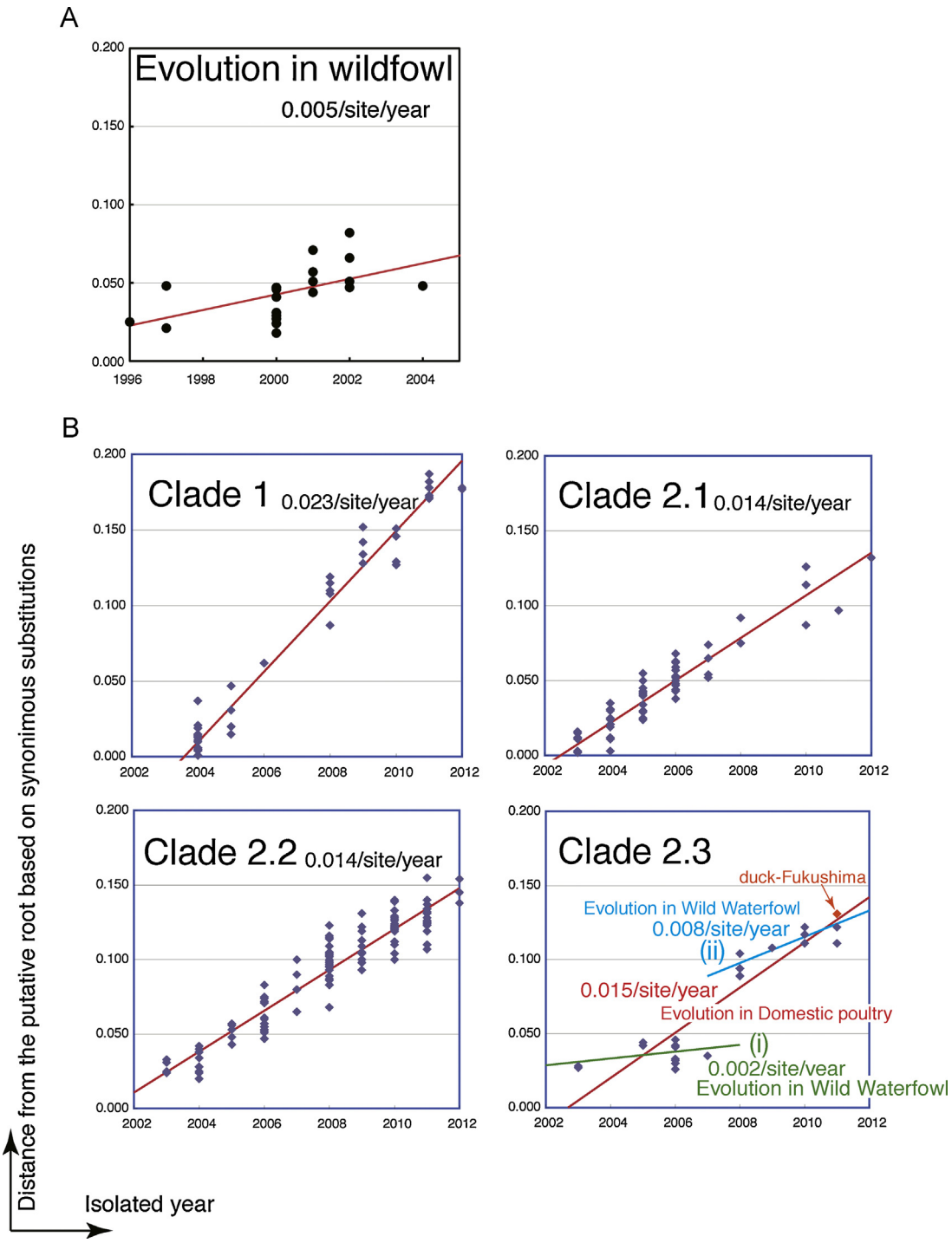


Fig. 1. Evolutionary rates of the hemagglutinin (HA) gene in H5N1 viruses. (A) Distances from the putative root for wildfowl viruses isolated between 1996 and 2004, plotted against isolation year. The evolutionary rate was estimated as 0.0050 substitutions per site per year. The viruses and the distances used for the calculation are listed in Supplemental Table 1. (B) Evolutionary rates of viruses from different clades. The distances that contribute to the graphs are shown in Supplemental Tables 2–5. The evolutionary trajectory of viruses belonging to subclade 2.3 is atypical compared with that of viruses in other clades. While the latter have evolved linearly (i.e., a constant rate of evolution over time, as shown red line), subclade 2.3 viruses have evolved discontinuously (green and blue lines). The duck-Fukushima strain, which belongs to subclade 2.3 (red symbol) was selected as the target for vaccine preparation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A FLAG tag (DYKDDDDK) was also added to the C-terminus of HA2 to label the modified HA protein (see Fig. 2). Because codon usage in silkworms is distinct from that in vertebrates, the nucleotide sequence of the H5HA gene was codon-optimized for silkworms. Because of these modifications to the original nucleotide sequence of the duck-Fukushima HA gene, the sequence homology between the authentic HA gene and the synthesized gene was 77.5% at the nucleotide level and 98.4% at the amino acid level. The synthesized gene was inserted into a baculovirus transfer vector, pBM-8, to obtain pBM-8-H5HA. Then, the recombinant baculovirus H5HA-BmNPV was generated via co-transfection of pBM-8-H5HA into Bm-N cells together

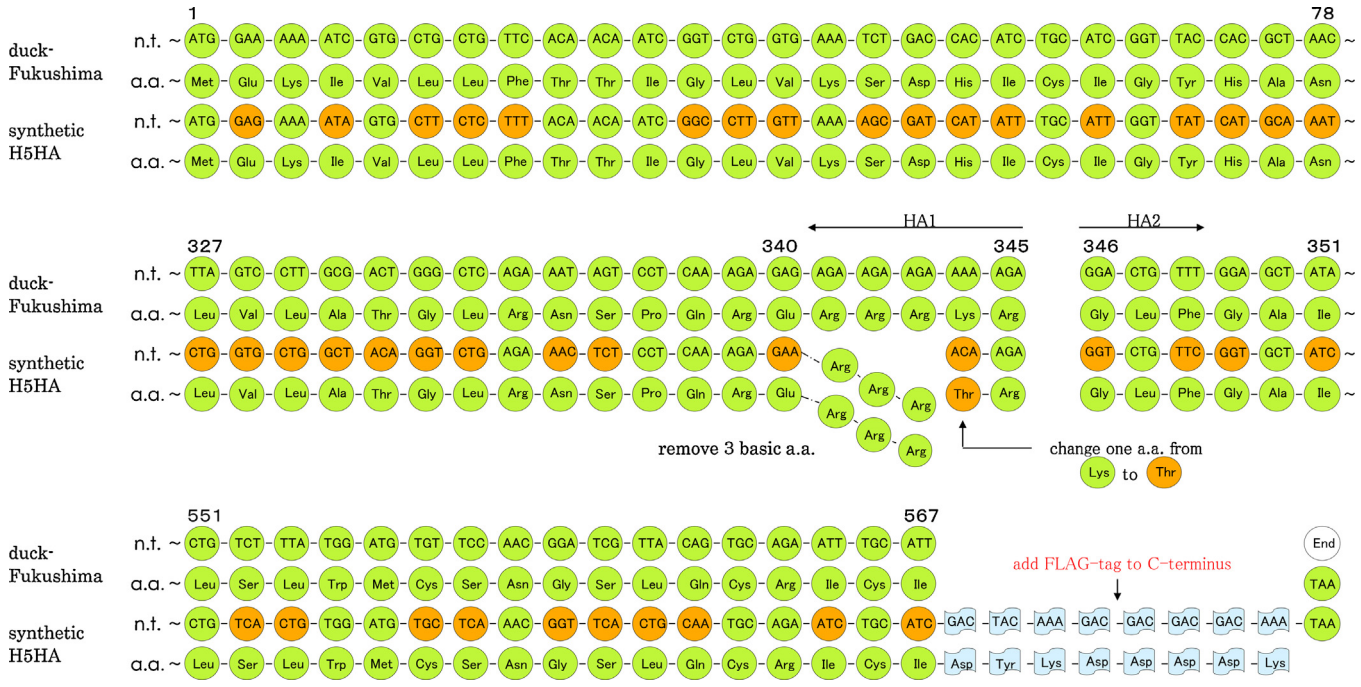


Fig. 2. Comparison of the nucleotide sequences for the authentic HA gene from the duck-Fukushima strain (light green) and the synthetic HA gene (orange). The codon usage of the H5HA gene was optimized for the silkworm, *B. mori*; the amino acid sequence connecting the H1 and H2 subunits (linked to the high pathogenicity of the duck-Fukushima strain) was changed from RERRRKRG to RDTRG, and a FLAG tag (DYKDDDDK) was added to the C-terminus of the H2 peptide. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with the linearized genomic DNA from the wild-type baculovirus BmNPV.

3.3. Expression of the synthetic H5HA gene in cultured cells

To examine the expression of the synthetic H5HA gene, we inoculated H5HA-BmNPV and wild-type BmNPV into Bm-N cells and performed a hemadsorption test on the following day. As shown in Fig. 3A, chicken red blood cells adhered to the recombinant virus-infected Bm-N cells (indicated by black arrows in Fig. 3A, H5HA-BmNPV) but not to mock-infected and wild-type virus-infected cells (BmNPV, Mock in Fig. 3A). Occlusion bodies were observed in the nuclei of BmNPV-infected cells (indicated by arrowheads in Fig. 3A, BmNPV), which were indicators of viral infection. In contrast, mock-infected Bm-N cells showed neither hemadsorption nor occlusion bodies.

In addition, an immunofluorescence test using serum from mice immunized with the homogenate of H5HA-BmNPV-infected pupae showed ring-shaped fluorescence in H5HA-BmNPV-infected Bm-N cells, but not in mock-infected and wild-type BmNPV-infected cells (Fig. 3B).

Western blot analysis of H5HA-BmNPV-infected Bm-N cells using an anti-FLAG antibody confirmed the expression of protein from the synthetic H5HA gene; a band of molecular weight 72.4 kD was clearly visible in lysates from H5HA-BmNPV-infected cells but not in those from wild-type BmNPV-infected cells (Fig. 3C). The expression of the H5HA protein in HeLa cells transfected with a pCAGGS expression vector [20] containing the synthetic H5HA gene was also confirmed by Western blot analysis (see Fig. 3C). The molecular weight of the H5HA protein in HeLa cells (78.3 kD) was greater than that in Bm-N cells. This difference may have been due to the structure of the carbohydrate chain attached to the HA protein, as the carbohydrate chain produced by insect cells is shorter than that produced by mammalian cells.

3.4. H5HA protein production in H5HA-BmNPV-infected pupae

To examine the potency of silkworm pupae as a protein production system, we injected H5HA-BmNPV into silkworm pupae. HA protein production was then monitored by testing homogenates of H5HA-BmNPV-injected pupae up to 4 days after injection. We found that HA titers increased linearly over the 4-day observation period, finally reaching approximately 1 million (Fig. 4A). Given this result, we thereafter harvested infected pupae at day 4. A homogenate of 220 pupae in approximately 200 mL (0.9 mL/pupa) PBS had an HA titer of 1,048,300. The amount of HA protein in an individual pupa (0.9 mL of homogenate with an HA titer of 1,048,300 is equal to 953,000 HAU) was more than 50 times higher than that obtained with an embryonated chicken egg, which generally produces approximately 10 mL allantoic fluid with an HA titer of 1,024–2,048, that is, 10,240–20,480 HAU/egg. Our rough estimation of the amount of HA protein produced by one silkworm pupa was 2,000 μg/pupa, which was based on the protein contents of the fractions with high HA titers obtained from sucrose density gradient centrifugation (see Fig. 5A).

To confirm the high level of HA protein production in pupae, we repeated the experiment twice using different numbers of pupae each time. When 47 and 158 pupae were homogenized in 50 mL and 150 mL PBS, respectively, 1115,000 HAU/pupa were obtained from the former and 497,000 HAU/pupa from the latter. The average HA protein production in the 3 experiments was 855,000 HAU/pupa (Fig. 4B). Even though the HA titers obtained from 3 experiments varied slightly, the amount of HA protein produced in a pupa were always much more than that produced in a chicken egg.

3.5. Immunogenicity of HA protein produced in pupae

To confirm the immunogenicity of the HA protein produced in silkworm pupae, we diluted the pupae homogenate in PBS to adjust the HA titer to 16,000. Then, 0.5 mL of the diluted homogenate was injected intradermally into 1-month-old female chickens, and

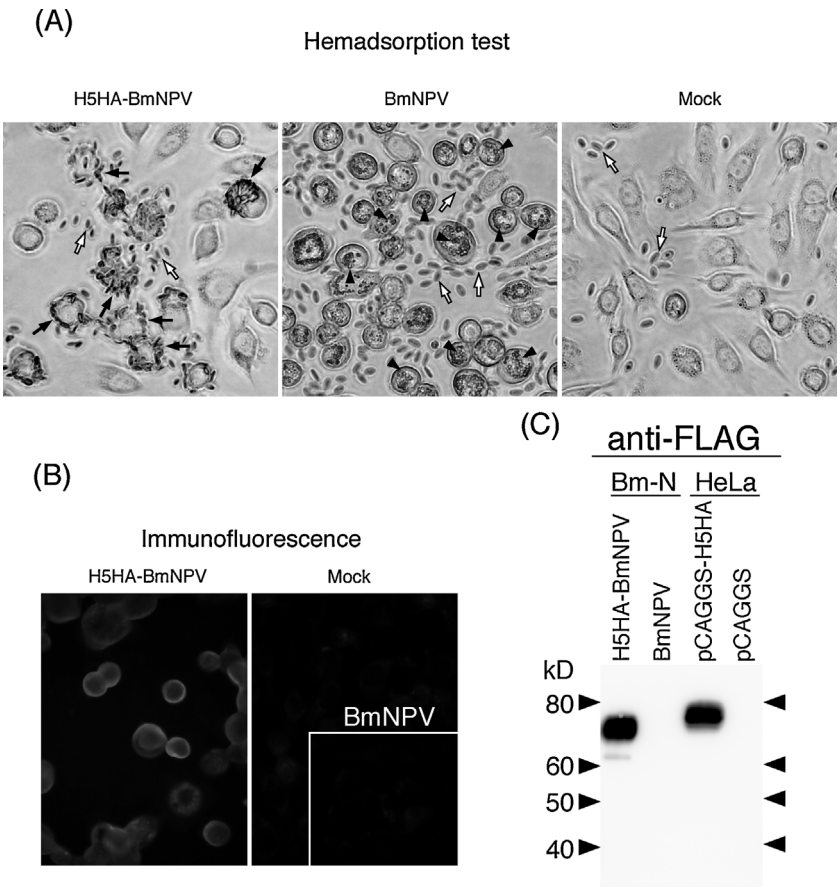


Fig. 3. Expression of the H5HA gene in H5HA-BmNPV-infected Bm-N cells. (A) Bm-N cells infected with H5HA-BmNPV and wild-type BmNPV were overlaid with chicken erythrocytes and incubated for 30 min at room temperature. Next, the cells were washed thoroughly with PBS and observed using phase-contrast microscopy. Mock-infected cells were also examined. Adsorption of chicken erythrocytes to H5HA-BmNPV infected cells was detected (black arrows). Occlusion bodies (arrow heads) were clearly visible in the nuclei of BmNPV-infected cells. Un-adsorbed erythrocytes (white arrows) were observed in H5HA-BmNPV, wild-type BmNPV, and mock-infected cells. Neither hemadsorption nor occlusion bodies were observed in mock-infected cell. (B) H5HA-BmNPV and mock-infected Bm-N cells were fixed and permeabilized. The cells were first incubated with serum from mice immunized with the homogenate of H5HA-BmNPV-infected pupae and then incubated with an FITC-conjugated anti-mouse IgG antibody. The stained cells were then observed by fluorescence microscopy. BmNPV-infected cells were also observed. (C) Bm-N cells were infected with H5HA-BmNPV or BmNPV. HeLa cells were transfected with pCAGGS containing the synthetic H5HA gene (pCAGGS-H5HA) or empty pCAGGS. The infected and transfected cells were then analyzed with Western blotting using an anti-FLAG tag antibody and a peroxidase-conjugated anti-mouse IgG antibody that was detected with an ECL detection system.

subsequent injections were performed on days 16 and 33 post-immunization (Fig. 4C). Their sera were subjected to HI tests on days 17, 31, 45, and 55 post-immunization. On each day, sera from 2 to 4 different chickens were prepared. As a result, an average HI titer of 128 was observed at 17 days after the first immunization, and high (4,096 and 3,327) titers were attained after 31 and 45 days. Furthermore, an HI titer of 1,448 was still observed even on 55 days post immunization. All chickens immunized with the VLP vaccine derived from silkworms did not show any side effects, including reduction in body weight. In contrast, unimmunized control chickens did not show any HI titers (data not shown).

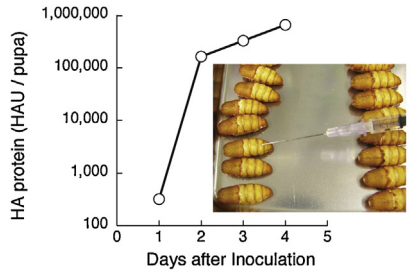
We determined the HI titer of the chicken serum against H5 influenza viruses including epizootic Indonesian viruses (Fig. 4D). We found that the serum reacted with all of the viruses examined. In addition, the serum inhibited plaque formation by the authentic A/duck/Singapore-Q/F119-3/97 (H5N3) virus (Fig. 4E).

3.6. *Analysis of homogenates from virally infected pupae using sucrose density gradient centrifugation*

To characterize the structure with hemagglutination activity, we fractionated the homogenate of H5HA-BmNPV-infected pupae and that of BmNPV-infected pupae using sucrose density gradient centrifugation and examined the HA titers and protein concentrations of the fractions. The analysis of H5HA-BmNPV-infected pupae showed that the protein concentration peaked in fractions 1, 25, and 40, which corresponded to 47.8%, 26.5%, and 10% sucrose, respectively, with the highest peak observed in fraction 40 (Fig. 5A). The HA titer showed 2 peaks at fractions 1 and 25 with the highest peak in fraction 25. On the contrary, none of the fractions of wild-type BmNPV-infected pupae had detectable HA titers and only 1 peak of the highest protein concentration was detected at fraction 36, corresponding to 10.2% sucrose (Fig. 5B). Based on these results, we assumed that fraction 25 of H5HA-BmNPV-infected pupae contained the H5HA protein. To confirm this assumption, Western blot analysis of fraction 25 was performed using anti-FLAG antibody. The analysis clearly showed a band of 72.4 kD, whereas the analysis of fraction 40 showed no clear band (Fig. 5C). Given these observations, we concluded that recombinant HA protein was concentrated in fraction 25 and its neighboring fractions (21–26) in the analysis of H5HA-BmNPV-infected pupae.

It is noteworthy that the density of fraction 25 was slightly less than that of the fraction in which intact influenza virions were detected. This was shown in the analysis of intact virions on the same sucrose density gradient where the peak HA titer was detected in the fraction corresponding to 38.5% sucrose (indicated by an arrow in Fig. 5A). The difference of density between VLP and intact virus may be attributable to the difference of protein

(A)

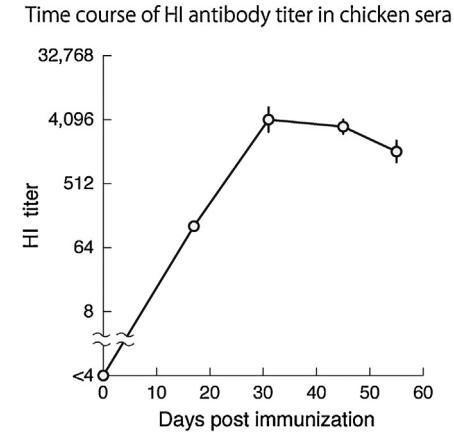


(B)

HA protein production in silkworm pupae

Experiment (no. of pupae)	HA protein at day 4 post inoculation (HAU / pupa)
1 (220)	953,000
2 (47)	1,115,000
3 (158)	497,000
Mean	855,000

(C)



(D)

HI titer of chicken serum against epizootic viruses

Antigen	HI titer
A/duck/Singapore-Q/F119-3/1997 (H5N3)	64
A/chicken/Legok/2004 (H5N1)	256
A/chicken/West Java/2009 (H5N1)	256
A/chicken/Kopal/2010 (H5N1)	512
H5HA-BmNPV infected pupae homogenate	2048

(E)

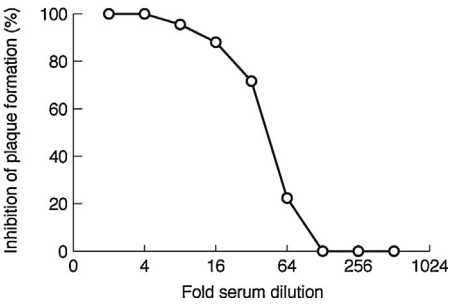


Fig. 4. Production of H5HA protein in silkworm pupae. (A) H5HA-BmNPV was injected into silkworm pupae. After the number of days indicated, the pupae were homogenized in PBS via sonication. The homogenate was centrifuged at 5,000 rpm for 30 min and then at 15,000 rpm for 30 min using an SW28 rotor. The HA titers of the supernatant were then determined. (B) A comparison of HA protein production levels from 3 experiments. For these experiments, H5HA-BmNPV-infected pupae were homogenized at day 4 post-injection. (C) The homogenate prepared from H5HA-BmNPV-infected pupae was diluted in PBS to an HA titer of 16,000. The diluted homogenate (0.5 mL) was injected subcutaneously into eight 1-month-old female chickens. The same amount was subsequently injected on days 16 and 33 after the first immunization. Blood was harvested on days 0, 17, 31, 45, and 55. Sera were prepared from the blood samples and used to determine the HI antibody titer against pupae homogenate. (D) The HI titer of chicken serum against epizootic H5 influenza viruses. The HI titer of the pooled serum of chickens immunized with H5HA-BmNPV-infected pupae homogenate on day 45 post-immunization was determined against various H5 influenza viruses. The HI titer against H5HA-BmNPV-infected pupae homogenate was also assayed. (E) A/duck/Singapore-Q/F119-3/97 (100 plaque forming units [PFUs]) was mixed with 2-fold serially diluted serum from chickens immunized with the homogenate of H5HA-BmNPV-infected pupae and then incubated for 30 min at room temperature. Then, the PFUs of each virus treated with serially diluted serum were determined. % inhibition = 100 × (100 − PFUs of treated virus)/100.

quantity between them. For example, the former contained only HA, but the latter was comprised of all viral proteins.

To characterize the structure with hemagglutination activity in fractions with high HA titers, we combined fractions 21–26 (see Fig. 5A) and analyzed them with negative-staining electron microscopy. The analysis revealed VLPs, as shown in Fig. 6A, C, and D. The particles had diameters of 30–300 nm and were covered with a large number of spikes. These spikes were approximately 14-nm long, similar to the previously reported lengths of influenza HA spikes [21]. The analysis of the fractions prepared from wild-type BmNPV-infected pupae revealed the presence of relatively small vesicles, but spikes similar to those on the surface of the VLPs were not observed (Fig. 6B).

The shape of the spikes was more clearly observed on the surfaces of VLP subpopulations with relatively low spike densities (see Fig. 6C). Triangular structures were discerned on the surfaces of the membranes (indicated by an arrow in Fig. 6D), consistent with the triple globular heads of HA trimers. In addition, rod-like shapes projecting from the VLP surfaces (two-headed arrows in Fig. 6A and arrowheads in Fig. 6D) were consistent with the side view of HA molecules. The subpopulations of VLPs (see Fig. 6C) that were approximately 100 nm in width resembled typical influenza viral particles of the H5N1 virus (A/HK/156/97) (see Fig. 6E). These structural characteristics observed in VLPs might be partly related to the condition of sonication process.

Although several previous reports have described the production of VLPs containing HA proteins using insect culture cells in which M1 and other genes were simultaneously introduced in addition to the HA gene [22–27], this is the first report demonstrating that the expression of the HA gene alone in silkworm pupae is sufficient to produce VLPs.

4. Discussion

For improvement of the immunogenicity of influenza subunit vaccines, the formation of VLPs by using a recombinant baculovirus expression system has been successfully performed in cultured insect cells [22–27]. In these previous studies, HA genes and other influenza genes (such as the M1 and NA genes) were co-expressed. By contrast, the system used in the present study, in which

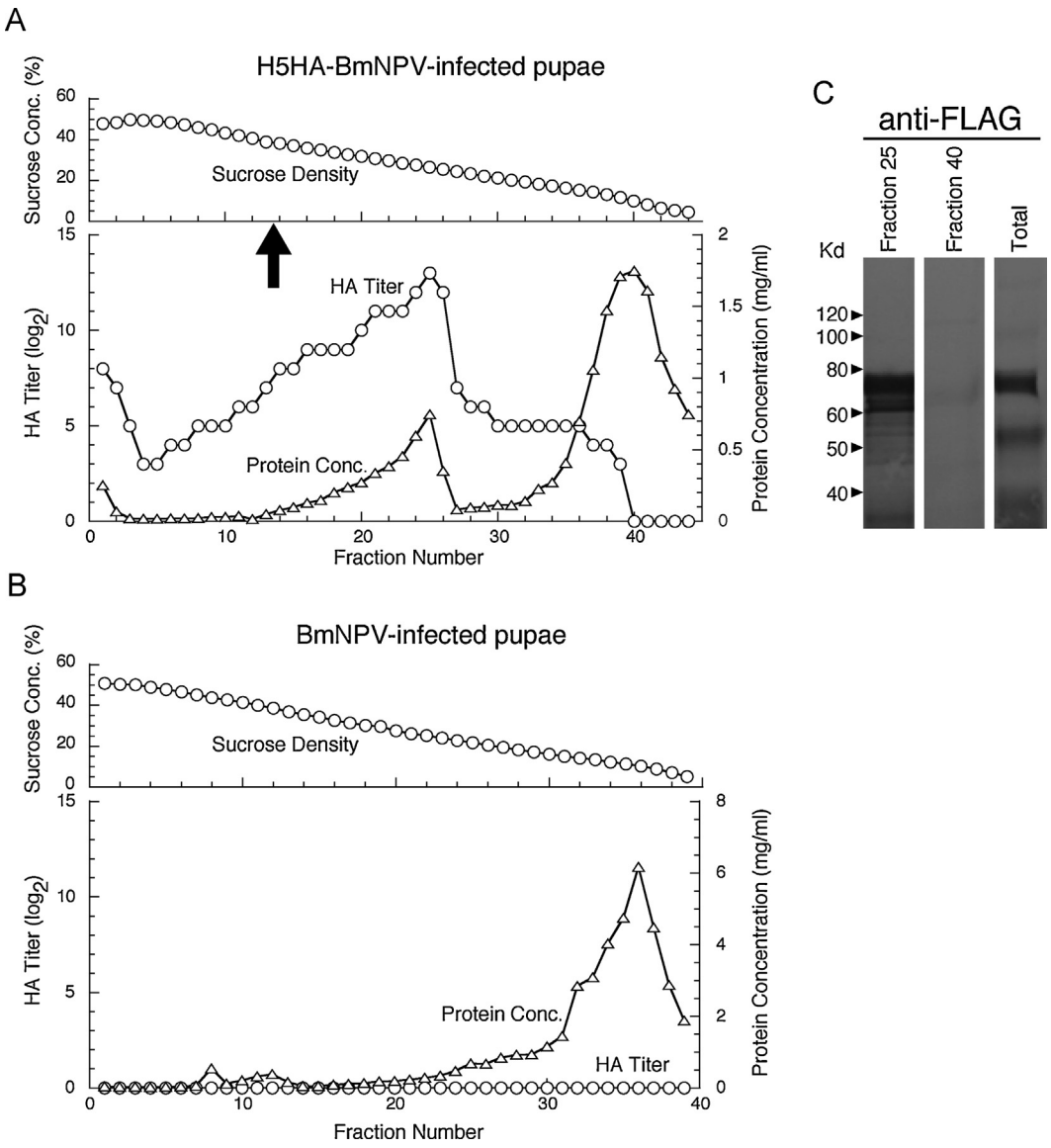


Fig. 5. Analysis of the homogenate of H5HA-BmNPV-infected pupae by using sucrose density gradient centrifugation. At day 4 post-infection, H5HA-BmNPV-infected (A) and wild-type baculovirus BmNPV-infected (B) pupae were homogenized in PBS via sonication. The homogenate was centrifuged using an SW28 rotor at 5,000 rpm for 30 min and then at 15,000 rpm for 30 min. The resulting supernatant was then applied to a 10–50% (w/w) sucrose density gradient and centrifuged at 25,000 rpm for 120 min. The gradient was fractionated from bottom to top, and the HA titer and protein concentration of each fraction were analyzed. The sucrose concentration of each fraction was also determined using a saccharometer. The fraction corresponding to the sucrose concentration where an HA titer peak was observed by the analysis of authentic influenza virus is indicated by an arrow. (C) Fractions 25 and 40 of H5HA-BmNPV-infected samples were analyzed with Western blotting using an anti-FLAG antibody. The homogenate of infected pupae was also analyzed.

silkworm pupae were used instead of cultured cells, produced a great deal of VLPs via the expression of a single synthetic HA gene. We assume that the high level of H5HA protein production observed in pupae is responsible for the efficient VLP formation using our system. Our preliminary study suggested an effect of codon optimization on the production yield of HA protein (at least the H5 and H7 subtypes) in the silkworm.

With respect to the purity of the HA protein in VLPs, 0.01 ng protein of the purified VLPs corresponded to 1 HAU based on the HA titer and protein content in fractions 21–26 in a sucrose density gradient analysis (Fig. 5 A). This estimated value indicated the high purity of VLPs prepared from silkworms.

Jin et al. produced a recombinant baculovirus displaying the H5HA protein (gp64HA fusion protein) on its surface using silkworm pupae, and 3% of the baculovirus protein was the fusion protein [28]. Dong et al. obtained approximately 500 μg of purified secretory H5HA protein (anchorless HA) from 30 silkworm larvae [29]. Similarly, Gomez-Casado et al. obtained 113 μg of H1HA protein from 1 g of fresh *Trichoplusia ni* larvae [30]. However, to the best of our knowledge, our present report is the first to describe the production of VLPs in insects via the expression of a single HA gene.

Recently, Giles et al. successfully developed an H5N1 VLP vaccine prepared from the supernatants of 293 T cells expressing NA, M1, and COBRA (computationally optimized broadly reactive antigen) HA genes [31]. This vaccine could produce effective and broad immune responses in nonhuman primates against H5N1 influenza viruses belonging to different evolutionary clades. If the COBRA HA could be produced in our system, the VLP HA vaccine may be of additional interest due to higher immune responses and the

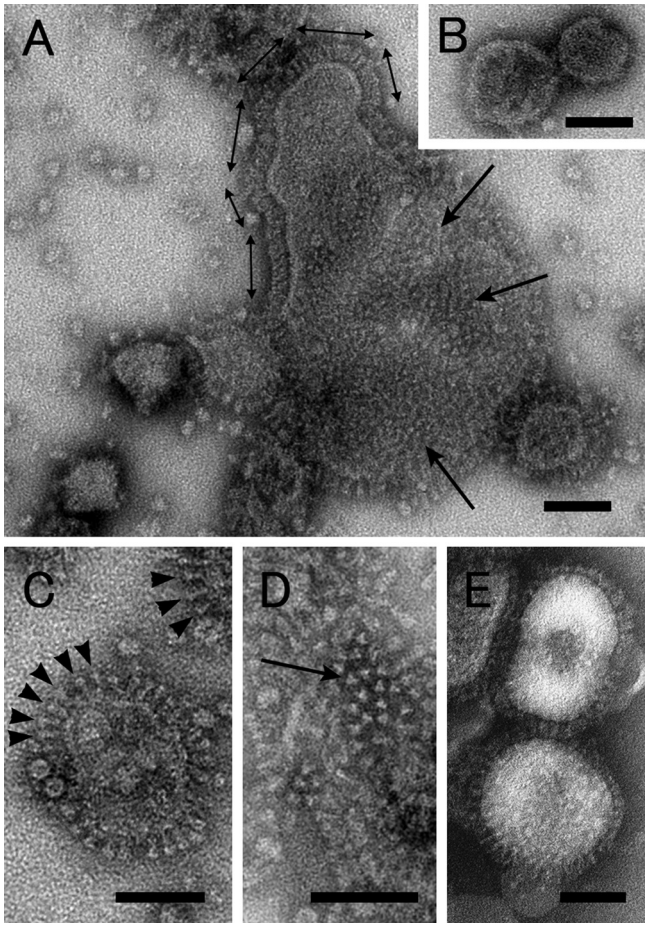


Fig. 6. Representative transmission electron microscopy images of virus-like particles produced by H5HA-BmNP-infected silkworm pupae. Combined fractions 21–26 from Fig. 5A were centrifuged at 25,000 rpm for 4 h. The resulting pellet was then resuspended in PBS and negatively stained with 2% phosphotungstic acid (pH 7) and observed using a TEM (Hitachi H-7600) operated at 80 kV. (A) Virus-like particles (VLPs) were pleomorphic and between 30 nm and 300 nm wide. Spike structures (two-headed arrows) approximately 14 nm long projected radially on the contours of the particles. The spike alignment was so dense that it appeared as an almost continuous layer. On the faces of the particles, a high density of small grains (arrows) were observed and attributed to the crowded heads of spikes 2-dimensionally arranged perpendicular to the plane of the micrograph. (B) Wild-type BmNPV-infected pupae were also analyzed using the same methods. In the preparation of BmNPV-infected pupae, vesicle-like structures were observed, but no spike was observed on their surfaces. (C, D) Examination of single spikes on subpopulations of VLP membranes, with occasional lower densities of spikes ascertained as rod-like shapes (arrowheads in C) and triangular heads (denoted by an arrow in D), consistent with the morphological features of HA spikes. (E) Viral particle images of authentic influenza strain A/HK/156/1997 (H5N1). Bar = 50 nm.

potential for mass production at a low cost. A series of studies to explore more appropriate antigens may contribute to future vaccine development.

In a previous study, we developed an influenza virosome vaccine with HANA using synthetic muramyl dipeptide [16]. Although the immunogenicity of this combination was higher than that of an ordinary embryonated egg-derived vaccine, inoculation with the vaccine resulted in local manifestations such as pain and erythema at the inoculation site. Because the system described in this study contains no artificial components, such side effects might be reduced. In fact, immunization with the VLP vaccine prepared in the present study did not induce clinical side effects and reduced the body weight of chickens and mice.

Concerning safety, allergic reactions caused by α1,3-linked fucose in the *N*-glycans of proteins produced in insect cells are important issues to be addressed in vaccine production using insects [32]. Recently, recombinant baculoviruses express enzymes that inhibit fucose addition to *N*-glycans have been successfully produced and are used to produce recombinant glycoproteins [33,34]. This approach has the potential to be integrated into our system.

In any case, our present study suggests the possibility of large-scale HA-VLP vaccine production in the near future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.11.009.

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