Biological and immunological characterization of influenza virus haemagglutinin expressed from the haemagglutinin locus of vaccinia virus

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Plasmid vectors were constructed to facilitate the insertion and expression of a foreign gene in the haemagglutinin (HA) gene locus of vaccinia virus. Five unique cloning sites adjacent to the P7-5 promoter of vaccinia virus permit the rapid insertion of a foreign sequence coding for a protein into these plasmids. This vector system provides a simple procedure to select recombinant viruses because they can be readily identified on the basis of their HA-defective phenotype. Recombinant vaccinia viruses expressing influenza virus HA were constructed to characterize the possible use of this system. The recombinant viruses did express the influenza HA through the authentic pathway of biosynthesis. In addition to having immunological characteristics similar to the authentic influenza HA, the expressed HA was found to possess haemagglutinating, haemadsorption and acid-inducible fusion activities. These findings demonstrate the usefulness of this eukaryotic vector system.

Introduction

Vaccinia virus is a member of the poxvirus family. There are a number of advantages of using this virus as a vector rather than other eukaryotic vectors such as simian virus 40, bovine papillomavirus, adenovirus, baculovirus and retrovirus vectors, because of its wide range of host species, high infectivity, high capacity to incorporate foreign genetic elements into the genome, no requirement for helper virus, and high ability to elicit humoral and cellular immune responses in experimental animals (for review, see Mackett & Smith, 1986). Numerous applications have demonstrated the suitability of vaccinia virus as a eukaryotic expression vector, and it has been shown that this vector system provides versatile tools to study the synthesis, intracellular localization and function of individual gene products. Particularly notable is a unique application to analyze target gene products recognized by cytotoxic T lymphocytes (Yewdell et al., 1985). Another important use of genetically engineered vaccinia viruses is in expressing protective antigens against specific pathogens as live vaccines (for review, see Moss & Flexner, 1987). It should be possible to prepare a polyvalent live vaccine capable of eliciting immune responses against multiple pathogens because of the capacity to retain multiple foreign genetic elements in the vaccinia virus genome (Perkus et al., 1985). Therefore, it should be worthwhile to investigate multiple sites where foreign genes can be inserted. The haemagglutinin (HA) gene of vaccinia virus is one site used for the insertion of foreign genes because it is not essential for virus replication (Ichihashi & Dales, 1971) and recombinant viruses with the foreign gene inserted into the HA gene can be readily selected by staining with chicken erythrocytes (Shida & Matsumoto, 1983). Indeed, recombinant vaccinia viruses with the envelope gene of human T cell leukaemia virus type I (HTLV-I) in the HA gene locus have already been constructed, and shown to induce humoral antibodies to HTLV-I and a protective effect against infection (Shida et al., 1987, 1988). However, the HA gene as an insertion site has not been well characterized, compared to the thymidine kinase (TK) gene locus in vaccinia virus.

In this communication, we describe plasmid vectors that facilitate the insertion and expression of a foreign gene in the HA gene of vaccinia virus. To characterize the system, we have employed cDNA copies of the HA gene of influenza virus as indicator genes and examined in detail the biological and immunological properties of the influenza HA protein expressed; several recombinant vaccinia viruses expressing the influenza HA genes from the TK gene locus have previously been constructed (Panicali et al., 1983; Smith et al., 1983, 1987; Boyle et al., 1985; Stephens et al., 1986; Rota et al., 1987; De et al., 1988; Chambers et al., 1988). Here we show not only faithful processing and intracellular transport of the HA
protein in cells infected with the recombinant vaccinia virus, but also its biological activities including haemagglutination, haemadsorption and cell fusion activities which have not been reported previously.

Methods

Cells and viruses. RK13, BHK-21 and CV-1 cells were grown at 37 °C in Eagle's MEM supplemented with 5% foetal calf serum (FCS). Vaccinia virus (strain WR) was propagated, titrated on monolayers of RK13 cells, and purified essentially as described by Joklik (1962). A vaccinia virus mutant dependent on isatin-β-thiosemicarbazone (IBT) was propagated as described previously (Fathi et al., 1986).

Construction of plasmid vectors pVR1 and pVR2 for insertion and expression of a foreign gene in the HA gene locus of vaccinia virus. Construction of plasmids was performed according to standard techniques (Maniatis et al., 1982). The 275 bp region of the HindII–Sai fragment containing the P7-5 promoter of vaccinia virus (which encodes a 7.5K polypeptide), was cloned into the HindII site of the plasmid pUC18 essentially according to Venkatesan et al. (1981). The resultant plasmid P7-5-2401 was digested with HindIII and EcoRI to obtain a fragment containing the P7-5 promoter element and multiple cloning sites placed adjacent to the promoter. This HindIII–EcoRI fragment derived from P7-5-2401 was isolated and treated with T4 DNA polymerase to create blunt ends. Plasmid pHA13, which encodes the vaccinia virus HA inserted between the Sall and HindIII sites of plasmid vector pUC13 (Shida, 1986), was cleaved with EcoRI and Sall, blunt-ended with T4 DNA polymerase and ligated with T4 DNA ligase to remove the multiple cloning sites. The resultant plasmid was designated pHA13.1. After plasmid pHA13.1 had been digested with Sall, it was ligated with the modified fragment derived from P7-5-2401. The resultant plasmids pVR1 and pVR2 contained the P7-5 promoter element and five unique restriction enzyme sites (BamHI, Sall, KpnI, ScaI, EcoRl) corresponding to the same (pVR1) or opposite (pVR2) direction to the flanking promoter of the vaccinia virus HA gene.

Construction of a series of insertion plasmids bearing the influenza virus HA gene for in vivo recombination. To construct infectious recombinant viruses, a series of insertion plasmids was constructed as follows. The 1.8 kb Sall fragment encoding HA of influenza virus strain A/Niigata/102/81 (H3N2 serotype) was isolated from the plasmid pSL30, which contains an HA gene cloned from a cDNA with the Sall linker into the plasmid vector pAT153 (S. Sakamoto, unpublished results). The isolated fragment was blunt-ended with T4 DNA polymerase and ligated with the plasmid vectors pVR1 or pVR2 which had been linearized with Sall and dephosphorylated with bacterial alkaline phosphatase. The resultant plasmids were classified according to the direction of the insert. Plasmids pVR1 and pVR2 contained the insert in the same orientation to P7-5 in the plasmid vectors pVR2 and pVR1, respectively. In contrast, plasmid pVR1 and pVR2 contained the insert in the opposite orientation to that in the plasmid vectors pVR2 and pVR1, respectively. A cDNA of the HA gene from A/SW/Cambridge/39 (H1N1) was cloned into the PstI site of the plasmid vector pUC119, using the dG-dC tailing procedure. The 5' end of this HA gene was trimmed with exonuclease III and mung bean nuclease to remove the 5' dG-dC tails, blunt-ended with the Klenow fragment of DNA polymerase, and then ligated with the BamHI linker. The new BamHI–PstI fragment encoding the HA gene of A/SW/Cambridge/39 was recloned into the plasmid vector pUC119 between the BamHI and PstI sites. Thus, the plasmid pHA 82.6 was obtained. Plasmid pHA 82.6 was digested with PstI and blunt-ended with T4 DNA polymerase. After digestion with BamHI, the 1.8 kb fragment was isolated and then ligated with the plasmid vector pVR2 which had been cleaved with BamHI and Sall. The resultant plasmid was designated pR-7.

Construction of infectious recombinant vaccinia viruses expressing the influenza virus HA gene. CV-1 cells (3 × 10^5 cells per 35 mm tissue culture dish) infected with vaccinia virus WR strain or the IBT mutant virus at 0.1 p.f.u. per cell were transfected with calcium phosphate-precipitated DNA of either vaccinia virus and an insertion plasmid or of an insertion plasmid alone. After 2 days, the cells were harvested, sonicated and plated on the monolayers of RK13 cells to give 500 to 1000 plaques per 100 mm tissue culture dish. After plaques had been stained with chicken erythrocytes, white plaques (HA^+) were picked as described previously (Shida & Matsumoto, 1983). The HA^+ plaques were plaque-purified twice before use. The recombinant viruses produced by transfection with pR-1, pR-2, pR-1r, pR-2r and pR-7 were designated vR-1, vR-2, vR-1r, vR-2r and vR-7, respectively. To confirm the expression of influenza virus HA by the recombinant viruses, vR-1, vR-2 and vR-7, plaques were incubated with the rabbit antiserum specific for the H3 or H1 subtype of HA, followed by peroxidase-conjugated goat anti-rabbit IgG antiserum (Cappel Laboratories) and finally developed with 4-chloro-1-napthol and hydrogen peroxide. To examine the presence of influenza virus HA gene DNA in the recombinant viruses vR-1r and vR-2r, dot-blot hybridization was done according to Mackett et al. (1985).

Immunoprecipitation analysis. CV-1 cells (3 × 10^5 cells in 35 mm tissue culture dish) were infected with the recombinant vaccinia virus at an m.o.i. of 10 or with influenza virus, and adsorbed for 1 h at 37 °C. The cells were washed twice with Eagle's MEM supplemented with 5% FCS and incubated at 37 °C for 4 h. After washing twice with methionine-free Eagle's MEM, the cells were treated with the same medium at 37 °C for 15 min. Then the cells were labelled for 3 h with 50 μCi of [35S]metionine (1000 Ci/mmol, Amersham) in methionine-free Eagle's MEM (100 μCi/ml) at 37 °C. The cells were rinsed three times with phosphate-buffered saline (PBS) and lysed in 0.5 ml of radioimmunoprecipitation (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM-PMSF, 100 units/ml aprotinin, 0.15 M-NaCl, 0.01 M-Tris-HCl pH 7.4). The lysate was incubated with rabbit antiserum against the H3 or H1 subtypes of influenza virus HA followed by incubation with Protein A-Sepharose CL-4B (Pharmacia). Otherwise lysate was incubated with mouse monoclonal antibodies against the HA of A/Niigata/102/81 or A/SW/Cambridge/39, and then with rabbit anti-mouse IgG antiserum followed by incubation with Protein A-Sepharose CL-4B. Immunoprecipitated polypeptides were resolved by 12% SDS-PAGE as described by Laemmli (1970). After electrophoresis, the gel was fixed and incubated with 1 mM-sodium salicylate for 30 min (Chamberlain, 1979), dried, and then exposed at −70 °C to RXO-H X-ray film (Fuji).

Haemagglutination assay. After infection with recombinant or wild-type vaccinia virus at an m.o.i. of 10, CV-1 cells were scraped off a tissue culture dish with a rubber policeman at the indicated times and centrifuged. Pelleted cells (10^7) were suspended in 200 μl of PBS and sonicated. The haemagglutination titre was determined in the homogenerate according to standard titration techniques using a microtitre plate.

Haemadsorption assay. At 24 h after infection with recombinant virus, monolayers of CV-1 cells were washed with PBS and overlayed with a 0.5% suspension of chicken erythrocytes in PBS. After 30 min the cells were washed three times with PBS and observed under a microscope.

Cell fusion assay. The cell fusion assay was carried out essentially according to White et al. (1981). At 24 h after infection with recombinant virus, BHK-21 cells were washed with Eagle's MEM and...
incubated with the former medium or with medium containing 10 µg/ml trypsin for 15 min at 37 °C. The cells were washed twice with medium supplemented with 5% FCS and incubated with fusion medium (RPMI 1640, 0.2% BSA, 10 mM-MES, 10 mM-HEPES pH 5) for 2 min at 37 °C. After removal of the fusion medium, the cells were incubated with medium supplemented with 5% FCS for 3 h at 37 °C. Then the cells were fixed and stained with Giemsa staining solution.

Antigenic characterization of influenza HA expressed by the recombinant vaccinia virus. Antigenic characterization was performed according to the standard haemagglutination inhibition test.

Endoglycosidase H digestion. Influenza HA expressed by the recombinant virus was labelled with [35S]methionine and immunoprecipitated as described above. Endoglycosidase H (endo H) digestion was done essentially as described previously (Copeland et al., 1986). The complex of immunoprecipitate with Protein A-Sepharose CL-4B was heated to 100 °C in SDS-PAGE sample buffer and the supernatant was recovered by centrifugation. The supernatant was then diluted 10-fold with 0.15 M-sodium citrate (pH 5.5), and added with 1 mM-PMSF and aprotinin (0.1 units/ml). After addition of endo H (50 milli-units/ml), the samples were incubated for 16 h at 37 °C. Samples were then precipitated with 10% TCA for 1 h at 0 °C, washed twice with cold ethanol:ether (1:1), and resuspended in SDS-PAGE sample buffer. The samples were subjected to 8% SDS-PAGE followed by fluorography as described above.

Results

Construction of plasmid vectors for insertion of a foreign gene into the HA locus of the vaccinia virus genome

To facilitate the insertion and expression of a foreign gene in the HA gene locus of vaccinia virus, we constructed two plasmid vectors, pVR1 and pVR2, as described in Methods (Fig. 1). These plasmids contain a vaccinia virus promoter element P7.5, five unique cloning sites for the insertion of a foreign gene, and flanking segments of the vaccinia virus HA gene. We chose the NruI site in the HA gene as an insertion site (Shida et al., 1987). Plasmids pVR1 and pVR2 can accommodate a foreign coding sequence under the control of P7.5 in the same and opposite orientation, respectively, to the HA gene promoter.

Construction of recombinant vaccinia viruses expressing influenza virus HA gene

To characterize this vector system, we used cDNA copies of the HA gene of influenza virus as indicator genes to construct recombinant vaccinia viruses. First, a series of insertion plasmids was constructed as described in Methods. Next we applied the method described by Fathi et al. (1986) to produce the recombinant vaccinia virus by using insertion plasmid pR-1. To compare the yield of recombinant virus, transfection was performed under two different protocols. CV-1 cells infected with wild-type or IBT® mutant vaccinia virus were transfected with calcium phosphate-precipitated DNA of either vaccinia virus and plasmid pR-1, or pR-1 alone. Subsequently the cells were harvested, and the collected viruses were plaque-assayed on RK13 cells. When plaques were stained with chicken erythrocytes, two types were observed as shown in Fig. 2(a and b). Since recombinant viruses that have an inserted foreign gene in the vaccinia virus HA gene locus result in the absence of HA activity, weak red plaques (HA-) were considered tentatively as recombinants. When cells infected with IBT® vaccinia virus were used as recipient cells for transfection, the percentage of HA- viruses in yielded virus increased 70-fold in contrast to WR-infected cells (data not shown). To confirm the HA- viruses as true recombinants, four plaques were picked up at random
from HA− plaques, and examined by staining with antibody specific for influenza virus HA. All the viruses were found to express influenza virus HA (data not shown). Accordingly we used this efficient protocol for subsequent construction of recombinant vaccinia viruses.

On screening HA− viruses, we found a difference in the HA− phenotype among recombinant viruses (Fig. 2). Plaques of vR-1 and vR-2 were slightly stained red by chicken erythrocytes, but plaques of vR-1r, vR-2r and vR-7 were not. To identify the HA− viruses as recombinants harbouring the influenza virus HA gene, we employed immunological staining or DNA hybridization methods. Thus a series of recombinant viruses carrying the influenza virus HA gene was constructed.

To characterize the structures of recombinant viruses, DNA from recombinant and wild-type vaccinia viruses was digested with HindIII, EcoRI or BamHI, and the DNA fragments separated by gel electrophoresis were transferred to a nitrocellulose filter and hybridized to 32P-labelled influenza virus HA cDNA (data not shown). The results confirmed the predicted structures of recombinant viruses. In addition, there were no obvious deletions or rearrangements of the recombinant genomes.

Expression of influenza virus HA by recombinant vaccinia viruses

To characterize the HA expressed by recombinant vaccinia viruses, CV-1 cells infected with the recombinant viruses were metabolically labelled with [35S]methionine. The influenza virus HA protein was immunoprecipitated with antiserum specific for influenza virus. In the lysate of cells infected with vR-1, a polypeptide of about 80K was specifically immunoprecipitated with antiserum against the H3 subtype of influenza virus, but not with that against H1 nor with the normal rabbit serum (Fig. 3). As a control, in the lysate of cells infected with vR-7, the anti-H1 serum immunoprecipitated polypeptides ranging from 75K to 80K, whereas the anti-H3 serum did not. Similar results were obtained from the immunoprecipitation experiment with monoclonal antibodies (MAbs) (data not shown). In addition, the 80K polypeptide immunoprecipitated from cells infected with vR-1 and vR-2 comigrated with authentic uncleaved influenza virus HA (HA0).

The influenza virus HA is known to have various biological activities which include haemagglutination, haemadsorption and membrane fusion. To demonstrate the biological activity of the HA expressed by recombinant vaccinia virus, haemagglutinating and haemadsorption activities were first examined. Haemagglutination was detected in homogenates of cells infected with vR-1, vR-2 and wild-type vaccinia virus (WR) (Fig. 4). However it was not detectable in the lysate from vR-1r- and vR-2r-infected cells. In each recombinant virus, the HA gene of vaccinia virus is inactivated by the insertion of the influenza virus HA gene. Therefore the result suggests that the haemagglutinating activity in cell homogenates from vR-1 and vR-2 could be attributed to influenza virus HA expressed by recombinant viruses, and not to vaccinia virus HA. To further clarify this result, a haemagglutination-inhibition test with anti-
Vaccinia virus expressing influenza virus HA

Fig. 3. Characterization of influenza virus HA expressed by recombinant vaccinia viruses. CV-1 monolayers were infected with vR-1 (lanes 1, 2 and 3) or vR-7 (lanes 4, 5 and 6) and labelled with [35S]methionine. The HA polypeptides were then immunoprecipitated with normal rabbit serum (lanes 1 and 4), the antiserum specific for H1 (lanes 2 and 5) or for H3 (lanes 2 and 6). Immunoprecipitates were then analysed by 12% SDS-PAGE and fluorographed. 14C-labelled proteins (Amersham) were used as M, standards.

Table 1. Antigenic characterization of HA produced by recombinant vaccinia viruses

<table>
<thead>
<tr>
<th>HA</th>
<th>MAb</th>
<th>Polyclonal antibody* to A/Niigata/102/81</th>
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<tr>
<td>vR-1</td>
<td>16000</td>
<td>1024</td>
</tr>
<tr>
<td>vR-2</td>
<td>16000</td>
<td>ND</td>
</tr>
<tr>
<td>A/Niigata/102/81</td>
<td>8000</td>
<td>2048</td>
</tr>
<tr>
<td>A/Bangkok/1/79</td>
<td>ND</td>
<td>512</td>
</tr>
<tr>
<td>A/Philippines/2/82</td>
<td>ND</td>
<td>128</td>
</tr>
<tr>
<td>A/Yamagata/96/85</td>
<td>ND</td>
<td>256</td>
</tr>
<tr>
<td>A/Fukuoka/C29/85</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Post-infection ferret serum.
† MAb INF-2 recognizes the HA of A/Niigata/102/81 but does not inhibit haemagglutination.
‡ < 1000.
§ ND, Not determined.

Fig. 4. Time course of HA activity in cells infected with the recombinant vaccinia viruses. CV-1 cells were infected with 10 p.f.u. of vR-1 (○, ●), vR-2 (□, ■), vR-1r (△, ▲), vR-2r (▽, ▼) or wild-type (WR) vaccinia virus (⊗, ♦) per cell in the presence (●, ■, ◆, ▼, ♦) or absence (○, □, △, ▽, ⊗) of 40 μg/ml ara C. At the indicated times after infection, the cells were removed and homogenized and the homogenates were assayed for haemagglutination titres. The HA titre is expressed as the reciprocal of the highest dilution of the homogenate (5 x 10⁶ cells/ml) causing haemagglutination.

Serum specific for influenza virus was done according to the microtitre method. The haemagglutinating activity of homogenates from vR-1 or vR-2 was inhibited by the antiserum specific for the H3 subtype of influenza virus (Table 1), but not by the antiserum to the H1 subtype or normal serum (data not shown). Similarly, MAbs specific for A/Niigata/102/81 inhibited the haemagglutinating activity of vR-1 and vR-2, and failed to react with vaccinia virus HA (Table 1). Fig. 5 shows the haemadsorption of infected cells with the recombinant or wild-type vaccinia virus. This result is consistent with the result obtained in the haemagglutination test.
The membrane fusion induced by influenza HA is known to depend on low pH and proteolytic cleavage of HA0 into HA1 and HA2 (White et al., 1981; Matlin et al., 1981; Huang et al., 1981; Maeda et al., 1981). Cell fusion occurred when BHK-21 cells infected with vR-1 or vR-2 were treated with trypsin and exposed to low pH (Fig. 6) and no cell fusion occurred without trypsin treatment. The low pH-dependent fusion activity was not observed in cells infected with vR-1r, vR-2r and wild-type vaccinia viruses.

To examine further whether these vaccinia virus vectors can successfully express the foreign gene product in a faithful manner, two different influenza HA species expressed by the recombinant vaccinia virus were monitored. The HA of A/SW/Cambridge/39 can induce membrane fusion, but not haemadsorption of chicken erythrocytes, whereas the HA of A/Niigata/102/81 retains both activities. Although both the recombinant viruses, vR-1 and vR-7 could induce cell fusion (Fig. 6a, g), haemadsorption was observed only in cells infected with vR-1 (Fig. 5a, e). The lack of haemadsorption of A/SW/Cambridge/39 was attributed to the intrinsic character of the HA, but not related to the neuraminidase activity associated with the virus. These results demonstrate that the biologically authentic influenza

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**Fig. 5.** Haemadsorption of CV-1 cells infected with recombinant vaccinia viruses. At 24 h after infection with vR-1 (a), vR-1r (b), vR-2 (c), vR-2r (d), vR-7 (e) or wild-type vaccinia virus (f), CV-1 cells were assayed for haemadsorption activity.

**Fig. 6.** Cell fusion induced by recombinant vaccinia viruses. At 24 h after infection with vR-1 (a and b), vR-2 (c and d), vR-2r (e and f), vR-7 (g and h), wild-type vaccinia virus (i and j) or influenza virus A/Niigata/102/81 (k and l), BHK-21 cells were treated with trypsin (a, c, e, g, i and k) or without trypsin (b, d, f, h, j and l). Then cells were incubated with the fusion medium and photographed with a microscope.
Vaccinia virus expressing influenza virus HA

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was investigated using endo H which removes N-linked carbohydrate chains of glycoprotein that have not been terminally glycosylated. When the immunoprecipitated HA from vR-1 was subjected to digestion with endo H, it was initially sensitive to endo H and gradually acquired resistance to it (Fig. 7). The deglycosylated size of the influenza virus HA from the recombinants is consistent with the predicted size. The acquisition by glycoprotein of endo H resistance is a marker for intracellular transport of the protein into or past the medial elements of the Golgi complex (Dunphy & Rothman, 1985). Cell surface expression of HA from recombinant vaccinia viruses was demonstrated by both immunofluorescence (data not shown) and haemadsorption experiments (Fig. 5). These findings indicate the faithful processing and intracellular transport of influenza virus HA expressed from recombinant vaccinia virus.

To analyse the kinetics of expression of the foreign gene by these vaccinia virus vectors, haemagglutinating activity was monitored in cells infected with the recombinants. The time course of HA activity is shown in Fig. 4. Haemagglutinating activity was first detected at 12 h after infection of vR-1 or vR-2, and continued to increase. No significant differences in HA synthesis between vR-1- or vR-2-infected cells were observed. This time course was similar to that for the expression of vaccinia virus HA, which is a late gene. In the presence of cytosine arabinoside (ara C), complete loss of HA synthesis was observed in cells infected with vR-1 and vR-2. Since ara C is an inhibitor of DNA replication, inhibition of HA synthesis by it implies that the polypeptide is synthesized late.

Discussion

In this communication, we describe the construction of plasmid vectors to facilitate the production and selection of infectious recombinant vaccinia viruses that express a foreign gene inserted in the HA gene locus, and properties of this vector system. These plasmids contain five unique cloning sites adjacent to the P7.5 promoter for the insertion of a foreign coding sequence, and flanking segments of the vaccinia virus HA gene for the recombination between homologous plasmid and viral DNA sequences. These vectors using the HA gene locus as an insertion site for the foreign gene enable simple selection of recombinant vaccinia virus, because the insertion of a foreign gene into the HA gene results in the inactivation of HA activity, and HA virus can be readily identified by staining with chicken erythrocytes (Shida & Matsumoto, 1983). This marker was still valid for screening recombinant vaccinia viruses expressing the influenza virus HA gene, because the difference in

virus HA was expressed through these vaccinia virus vectors.

The antigenic properties of HA expressed by the recombinant viruses were evaluated using MAbs and post-infection ferret serum to A/Niigata/102/81 (Table 1). All the tested epitopes present on native influenza HA were detected on the HA synthesized by vR-1 and vR-2. The total antigenic structure of recombinant HA recognized by ferret serum was similar to that of the authentic one.

Influenza virus HA is assembled into a trimer, glycosylated and finally transported to the cell surface (Gething et al., 1986; Copeland et al., 1986, 1988; Yewdell et al., 1988). When the HA is electrophoresed on an SDS–polyacrylamide gel without heat treatment and in the absence of reducing agent, three bands corresponding to monomer, dimer and trimer are detected (Doms & Helenius, 1986). Fig. 7 shows the formation of trimer HA expressed by vR-1. Glycosylation of influenza virus HA expressed from the recombinant vaccinia virus

Fig. 7. Analysis of glycosylation and trimer formation of influenza virus HA expressed by recombinant vaccinia virus. (a) At 4 h after infection with vR-1 (all lanes), CV-1 cells were labelled with [35S]methionine for 1 h (lanes 1 and 2) or 3 h (lanes 3 and 4) and lysates were immunoprecipitated with specific antibody. Immunoprecipitated material was incubated in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of endo H and analysed by SDS-PAGE followed by fluorography. (b) CV-1 cells were infected with vR-1 and labelled with [35S]methionine. Immunoprecipitates were solubilized in SDS-PAGE sample buffer without 2-mercaptoethanol at 37 °C, and analysed by SDS-PAGE and fluorography. 14C-labelled proteins (Amersham) were used as Mr standards.
the HA-defective phenotype could be observed between recombinant and non-recombinant viruses (Fig. 2). However there was an apparent discrepancy in the haemadsorption activity of expressed influenza virus HA between low and high m.o.i. conditions (Fig. 2 and Fig. 5). This may be explained as different kinetics of expression of HA activity in the cells infected with recombinant and wild-type vaccinia virus (Fig. 4). Indeed, only in the late phase after infection (over 20 h) could we detect significant haemadsorption activity induced by recombinant viruses, even at a high m.o.i., in contrast to wild-type virus (Fig. 5 and data not shown). As shown in Fig. 2, the plaques formed by the recombinant virus showed weak haemadsorption.

Vaccinia virus vectors have been successfully used as eukaryotic expression vectors. We have shown that the recombinant vaccinia viruses could express trimerized influenza HA through the authentic pathway of biosynthesis; the HA expressed possessed haemagglutinating, haemadsorption and acid-inducible fusion activities in addition to authentic antigenicity. We also demonstrated the faithful expression of influenza HA derived from two different strains, clarifying the unique character of HA from A/SW/Cambridge/39, which lacks the haemadsorption activity in spite of the presence of membrane-fusing activity. Although several recombinant vaccinia viruses expressing influenza virus HA have been constructed and characterized previously (Panicali et al., 1983; Smith et al., 1983, 1987; Boyle et al., 1985; Stephens et al., 1986; Rota et al., 1987; De et al., 1988; Chambers et al., 1988), the HA expressed was assayed only for its glycosylation, localization and antigenicity.

The expression of influenza virus HA has been conducted in several heterogeneous systems. The results reveal that the expression systems of Escherichia coli and yeast are not suitable for the production of authentic HA in large quantities (Emtage et al., 1980; Heiland & Gethin, 1981; Davis et al., 1981; Jabbar et al., 1985). Baculovirus vectors could express HA which has full biological activities but is glycosylated abortively (Kuroda et al., 1986). On the other hand, although authentic influenza virus HA is expressed in a large quantity by the simian virus 40 vector (Gethin & Sambrook, 1981), the limitations of this vector system including the requirement for a helper virus and narrow host range may reduce its usefulness. Therefore, these vaccinia virus vectors offer a useful system to study biosynthesis and biological activities of glycoproteins seen by our demonstration of the synthesis of fully active HA. Recently, the strong promoter of the A-type inclusion body of cowpox virus has been isolated and sequenced (Funahashi et al., 1988); modification of the vectors with this promoter will enhance their use in future.

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