JOURNAL OF VIROLOGY, June 1993, p. 3069–3076
0022-538X/93/063069-08$02.00/0

Vol. 67, No. 6

Fowlpox Virus Recombinants Expressing the Envelope Glycoprotein of an Avian Reticuloendotheliosis Retrovirus Induce Neutralizing Antibodies and Reduce Viremia in Chickens

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Received 23 November 1992/Accepted 1 March 1993

Eight stable fowlpox virus (FPV) recombinants which express the envelope glycoprotein of the spleen necrosis virus (SNV) strain of reticuloendotheliosis virus (REV), an avian retrovirus, were constructed. These recombinants differ in the genomic location of the inserted genes, in the orientation of the insert relative to flanking viral sequences, and in the promoter used to drive expression of the *env* gene. Of these variables, promoter strength seems to be the most crucial. The $P_{7.5}$ promoter of vaccinia virus, which is commonly used in the construction of both vaccinia virus and FPV recombinants, resulted in lower levels of expression of the envelope antigen in infected chicken cells compared with a strong synthetic promoter, as determined by immunofluorescence and enzyme-linked immunosorbent assay. Two peptides encoded by the *env* gene, the 21-kDa transmembrane peptide and a 62-kDa precursor, were detected by immunoprecipitation of labeled proteins from cells infected with recombinant FPVs, using monoclonal antibodies against REV. These peptides comigrated with those precipitated from REV-infected cells. One of the recombinants (f29R-SNenv) was used for vaccination of 1-day-old chickens. Vaccinated chicks developed neutralizing antibodies to SNV more rapidly than did unvaccinated controls following SNV challenge and were protected against both viremia and the SNV-induced runting syndrome.

The reticuloendotheliosis viruses (REVs) are a group of oncogenic and immunodepressive type C avian retroviruses (51). They are distinct from the avian leukosis/sarcoma virus group (21) and are more closely related to mammalian retroviruses, both antigenically (2, 46, 47) and at the genome level (26, 35). Nondefective strains of REV include REV-A, spleen necrosis virus (SNV), chicken syncytial virus, duck infectious anemia virus, and a number of other isolates (12). These nondefective REVs cause a runting disease syndrome characterized by splenomegaly, necrosis of the spleen and liver, nerve lesions, and B- or T-cell lymphomas in chickens. A single replication-defective, acutely transforming REV isolate (REV-T) is known; this isolate carries the *rel* oncogene and requires a nondefective helper virus (such as strain REV-A) for replication. REV-T causes an acute reticulum cell neoplasia in inoculated chickens. REV causes immunodepression in infected chickens and has been found as a contaminant of Marek's disease (20, 54) and fowlpox (5) vaccines. REV is associated with sporadic outbreaks of chronic neoplastic disease in turkeys and can cause significant losses in turkey flocks (51, 52). No vaccine for REV is currently available. The oncogenic potential of these viruses, their ability to cause immunodepression, and their presence as contaminants in poultry biologics warrant development of a suitable vaccine.

The envelope glycoproteins of retroviruses (encoded by the *env* genes) are known to be associated with virus neutralization. The various strains of REV are antigenically very similar, and antibody against each strain cross-neutralizes other strains (12), suggesting that a live vaccine expressing the *env* gene of a single REV isolate may provide protective immunity against numerous REV-associated diseases. The genomes of SNV and REV-A have been molecularly cloned (11, 34). Sequence analysis of the *env* genes of these viruses shows that they are 92.7% identical at the amino acid level and about 40 to 50% identical to the *env* genes of type D and some type C simian retroviruses, with which they share a receptor (22, 23).

The poxviruses have attracted much attention in recent years as vectors for the expression of foreign genes and for the construction of potential vaccines against animal diseases (6, 39). This interest is primarily due to their high capacity for accepting foreign DNA and to their cytoplasmic replication site. Most of this attention has focused on vaccinia virus, the prototype of the genus *Orthopoxvirus* (19, 27, 29), because of its wide host range and relatively well defined molecular biology (18, 28).

Members of the genus *Avipoxvirus* have a host range which is restricted to avian species. Attenuated vaccine strains of these viruses are commercially available (45). Avipoxviruses show promise not only as safe vectors for live recombinant poultry vaccines but also as vectors for replication-defective mammalian vaccines (41, 42, 44, 49). Fowlpox virus (FPV), the prototype of this genus, has been used successfully as a recombinant vaccine to immunize chickens against several diseases, including Newcastle disease (7, 8, 17, 25, 33, 40), avian influenza (4, 9, 43), Marek's disease (31, 53), and infectious bursal disease (3).

We have inserted the *env* gene of SNV, under the control of either $P_{7.5}$ or a strong synthetic poxvirus promoter, into either of two nonessential positions in the FPV genome, in both orientations. Of these eight recombinants, the four which employed the synthetic promoter gave much higher levels of envelope expression, as determined by immunofluorescence and enzyme-linked immunosorbent assay (ELISA),

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and were further characterized by radioimmunoprecipitation. One recombinant was tested in challenge experiments and found to induce protective immunity against SNV challenge. These recombinant FPVs may prove useful as vaccines against REV-induced diseases.

MATERIALS AND METHODS

Cells, viruses, plasmids, and monoclonal antibodies (MAbs). Primary chicken embryo fibroblasts (CEF) were prepared from 11-day-old RPRL line 0 White Leghorn embryos by the method of Solomon (37). CEF were grown in Leibovitz-McCoy medium supplemented with 4% calf serum and antibiotics.

A large-plaque variant of FPV (30), originally derived from a vaccine strain (CEVA Laboratory, Overland Park, Kans.), was used in the generation of recombinants. Two randomly selected nonessential sites on DNA fragments from the NP vaccine strain (Shionogi & Co. Ltd., Shiga, Japan) of pigeon pox virus (PPV) were used in the construction of plasmid transfer vectors. Homologous recombination between PPV plasmids and FPV occurred readily, since the two viruses are very closely related. The restriction enzyme cleavage patterns of PPV are similar to those of FPV (33, 36), and sequencing of over 5 kb of homologous DNA indicates that PPV and FPV genes are about 99.9% identical (unpublished data).

The SNV and REV-T(F) strains of REV (12, 51) were used in these studies.

Plasmid pPB101, which contains the complete provirus of the SNV strain of REV cloned into pBR322 (1), was a generous gift from Howard M. Temin (McArdle Laboratory, University of Wisconsin, Madison).

MAbs 11A25 and 11B118 (13), prepared against REV strain T(C), were kindly provided by Lucy F. Lee (USDA-ARS Avian Disease and Oncology Laboratory, East Lansing, Mich.). Both of these MAbs recognize the transmembrane portion of the REV envelope glycoprotein.

Construction of plasmid vectors. A 1,829-bp *Bam*HI-*Sac*I fragment of pPB101, which contains the entire open reading frame for the SNV envelope glycoprotein as well as 46 upstream and 41 downstream nucleotides, was initially cloned into a small adaptor plasmid and subsequently into eight related insertion vectors (designated pNZ1729R, pNZ1729L, pNZ1725R, pNZ1725L, pNZ1629R, pNZ1629L, pNZ1625R, and pNZ1625L) for recombination into FPV (Fig. 1). The construction of pNZ1729R, which directs the insertion of foreign genes, driven by a synthetic strong early/late poxvirus promoter (P_s), into a nonessential site of the FPV genome designated position 29, has been described elsewhere (53). A different nonessential site, designated position 25 (32), is targeted by pNZ1725R and other "25" plasmids. The orientation of the inserted foreign gene-*lacZ* cassette is reversed in pNZ1729L and other "L" plasmids, relative to surrounding FPV sequences. Plasmids which contain the "16" designation use the vaccinia virus $P_{7.5}$ promoter to drive expression of the foreign gene, while those with the "17" designation use the P_s synthetic promoter.

Generation and purification of recombinant FPV. CEF monolayers (~10^7 cells) were infected with FPV at a multiplicity of infection of 0.1 and incubated for 5 h in serum-free medium. Cells were trypsinized, washed twice in saline G (0.14 M NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.011% glucose), and resuspended in 0.7 ml of saline G. This cell suspension was mixed with 10 μg of CsCl-purified transfer vector DNA in 0.1 ml of saline G

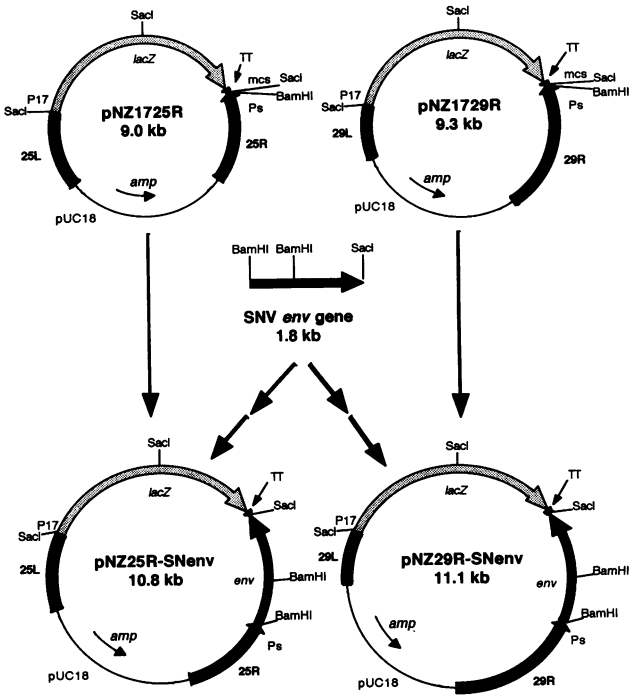

FIG. 1. Construction of plasmid vectors for recombination into FPV. The *env* gene of SNV was excised from pPB101 (1) by complete digestion with *Sac*I and partial digestion with *Bam*HI. The 1,829-bp fragment was cloned, in a two-step procedure, into eight plasmid vectors as described in Materials and Methods. Two of these plasmids (pNZ1725R and pNZ1729R) are shown. The resulting chimeric plasmids (pNZ25R-SNenv and pNZ29R-SNenv) were used to construct recombinant FPVs. Abbreviations: *lacZ*, *E. coli* β-galactosidase gene; *amp*, ampicillin resistance gene from pUC18; *env*, SNV envelope glycoprotein gene; TT, synthetic bidirectional poxvirus early transcriptional terminator; mcs, multiple cloning site; P_s, synthetic strong late/early poxvirus promoter; P_{17}, a moderately weak FPV early promoter; 25R, 25L, 29R, 29L, the right (R) and left (L) arms of poxvirus DNA flanking the nonessential sites 25 and 29.

and subjected to electroporation at 300 V (750 V/cm), 330 μF, and low Ω, using a Cell-Porator apparatus (GIBCO/BRL, Gaithersburg, Md.) at room temperature. Transfected cells were plated onto a single 60-mm-diameter culture dish and incubated at 37°C. After 3 days, these cells were harvested by scraping and disrupted by sonication to release progeny virus.

Recombinant FPVs were identified and purified by using a modification of the method described by Dhawale et al. (16). Briefly, dilutions of the cell lysates were assayed on CEF with an overlay of medium containing 0.8% Bacto Agar and lacking phenol red pH indicator. Total plaques were counted after 5 to 7 days. Recombinants, which expressed the *lacZ* gene, were identified by staining with 5 ml of a second agar overlay which contained 1 mg of Bluo-gal (GIBCO/BRL) per ml and lacked serum. Blue plaques became apparent after 1 to 3 days. Several blue plaques were picked and placed into a small volume of medium, disrupted by sonication, and used to infect the next round of cultures. Plaque purification was continued until only blue plaques were detected.

Recombinant viruses were named according to the transfer vector used (for example, the recombinant virus f29R-SNenv was constructed by using plasmid pNZ29R-SNenv).

TABLE 1. Detection of SNV envelope glycoprotein in recombinant FPV-infected cells by immunofluorescence and by ELISA

Virus	Genomic position	Orientation	Promoter	Fluorescence intensity[a]		ELISA[b]
				Expt 1	Expt 2	
$f_{7.5}$29R-SNenv	29	Right	$P_{7.5}$	+	+	0.308 ± 0.192
$f_{7.5}$29L-SNenv	29	Left	$P_{7.5}$	+	+	0.320 ± 0.186
$f_{7.5}$25R-SNenv	25	Right	$P_{7.5}$	+	+	0.362 ± 0.249
$f_{7.5}$25L-SNenv	25	Left	$P_{7.5}$	+	+	0.299 ± 0.198
f29R-SNenv	29	Right	P_s	++++	+++	1.206 ± 0.060
f29L-SNenv	29	Left	P_s	++++	+++	1.167 ± 0.098
f25R-SNenv	25	Right	P_s	+++	+++	1.280 ± 0.034
f25L-WNenv	25	Left	P_s	+++	+++	1.280 ± 0.041
Parental FPV	NA[c]	NA	NA	−	−	−0.041 ± 0.107
None	NA	NA	NA	NA	NA	0.000 ± 0.069

[a] Expression of the SNV *env* gene in infected CEF was assayed by indirect immunofluorescence using anti-REV chicken sera (experiment 1) or MAb 11A25 (experiment 2) as described in Materials and Methods. Relative fluorescence intensity of cells within FPV plaques was evaluated on a scale ranging from + (faintly fluorescent) to ++++ (brightly fluorescent), with − indicating nonfluorescent plaques.

[b] Expression of the SNV *env* gene in infected CEF was quantified by ELISA as described in Materials and Methods. Data are the average OD_{490} values (±1 standard deviation) from eight identical wells normalized to the average of eight uninfected control wells.

[c] NA, not applicable.

The structural characteristics of the eight FPV recombinants are shown in Table 1.

Southern hybridization analysis. Total DNA was extracted from CEF infected with recombinant or parental FPV at 4 days postinfection, using proteinase K digestion in the presence of sodium dodecyl sulfate (SDS) and EDTA, followed by phenol-chloroform extraction and ethanol precipitation (10). After digestion with appropriate restriction enzymes, the DNA was separated on agarose gels and transferred to Zeta-Probe membranes (Bio-Rad, Richmond, Calif.). Labeling of probes and chemiluminescent detection of hybridization signals was performed by using the Genius/Lumiphos system (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the manufacturer's instructions, except that 3% SDS was used in the prehybridization and hybridization solutions.

Indirect immunofluorescence. Indirect fluorescent antibody assays were performed on recombinant FPV-infected CEF grown on glass coverslips (50). A pool of anti-REV (strain T) sera from convalescent chickens or MAb 11A25 (13) was used as the primary antibody. Fluorescein isothiocyanate-conjugated rabbit anti-chicken immunoglobulin G (IgG) or fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cappel, Durham, N.C.) was used as the secondary antibody. Plaques were visualized by using a dark-field microscope with UV (ploem) illumination.

ELISA. Expression of the SNV envelope glycoprotein was quantified by ELISA performed on recombinant FPV-infected CEF. Freshly prepared and titrated stocks of the eight recombinant FPVs and parental FPV were diluted in medium to a concentration of 2×10^6 PFU/ml, and 100-μl aliquots were dispensed into eight wells each of a 96-well microtiter plate. To these wells was added 100 μl of medium containing 5×10^5 trypsinized CEF per ml (multiplicity of infection of 4). Infection was allowed to proceed at 37°C for 16 h, at which time cultures were rinsed with phosphate-buffered saline (PBS), fixed in acetone-ethanol (3:2) for 5 min, and allowed to dry.

The fixed cells were then rehydrated in PBS and allowed to react for 1 h with MAb 11A25 (13) (100 μl per well) diluted 1:100 in PBS containing 3% bovine serum albumin and 0.1% sodium azide. Antibody was removed, and wells were rinsed twice with PBS. Cells were then reacted for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) (100 μl per well) diluted 1:500 in PBS. After two washes with PBS, 100 μl of substrate solution containing 800 μg of 5-aminosalicylic acid per ml and 0.168 μl of 30% H_2O_2 solution per ml in 0.2 M sodium phosphate buffer (pH 6.0) was added to each well.

The substrate reaction was allowed to proceed overnight at room temperature in the dark. Cells were solubilized by the addition of 100 μl of 1% SDS in 200 mM Tris-HCl (pH 8.0), followed by vigorous mixing with a multichannel micropipet. Optical densities at 490 nm (OD_{490}) were determined with an ELISA reader and normalized to the average of eight wells containing uninfected cells.

Radioimmunoprecipitation. ^{35}S-labeled envelope glycoprotein was immunoprecipitated from infected cell lysates with MAbs, using a modification of the procedure of Cui et al. (13). CEF monolayers on 60-mm-diameter tissue culture plates were infected with recombinant or parental FPV at a multiplicity of 5 PFU per cell or with REV strain SNV or REV-T at a multiplicity of 0.1 focus-forming units (FFU) per cell or were mock infected. FPV and mock-infected plates were labeled for 5 h at 20 to 25 h postinfection. REV-infected cells were labeled for 5 h on day 6 postinfection. Medium was replaced with 2 ml of methionine-free medium 1 h prior to the labeling. Metabolic labeling was with 40 μCi of [^{35}S]methionine/[^{35}S]cysteine (Tran^{35}S-Label; ICN Biomedicals, Costa Mesa, Calif.) per ml. Cells were then washed thoroughly in PBS, scraped and pelleted, resuspended in 300 μl of lysis buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris-HCl [pH 7.5]), and allowed to sit for 30 min at room temperature. Lysates were stored at −20°C until needed.

Portions (100 μl) of the lysates were preabsorbed with normal mouse ascites fluid and *Staphylococcus aureus* Cowan I cells (Boehringer Mannheim). Supernatants were incubated with 2 μl of ascites fluid containing MAb 11A25 or 11B118 (13) and precipitated with *S. aureus* Cowan I cells. After thorough washing with lysis buffer, the pellets were resuspended and boiled in 35 μl of electrophoresis sample buffer, and 20 μl was run on SDS-12% polyacrylamide gels (24). Following electrophoresis, gels were fixed, impregnated with 22% 2,5-diphenyloxazole in dimethyl sulfoxide, soaked in water, dried, and exposed to X-ray film (Kodak XAR 5).

Protection against SNV challenge. Groups of $15I_5 \times 7_1$ (38) White Leghorn chicks ($15I_5 \times 7_1$ is a cross between two B-congenic lines, $15I_5$ males $\times$ 7_1 females) were vaccinated intra-abdominally at 1 day of age with 10^6 PFU of recombinant f29R-SNenv or parental FPV in a volume of 0.1 ml (13 to 15 birds per group). At 2 weeks of age, they were challenged intra-abdominally with 2×10^3 FFU of SNV in 0.1 ml. Birds were bled on the day of challenge and periodically thereafter. Plasmas from individual birds were titrated for viremia, and pooled plasma samples (from all birds in a group) were inactivated (30 min at 56°C) and assayed for neutralizing antibody against SNV.

For neutralization assays, equal volumes of serum from each bird in a group were pooled, and appropriate dilutions of the pools were made in 100 μl of cell culture medium. Approximately 100 FFU of SNV in 100 μl of medium was added to the dilutions, and neutralization was allowed to proceed for 30 min at room temperature. The virus titer was then determined by using an indirect immunoperoxidase focus reduction assay which yields macroscopic foci in tissue culture dishes (unpublished data). Briefly, infected CEF monolayers in 60-mm-diameter dishes were initially overlaid with 4 ml of 0.6% Bacto Agar in medium. After 3 days, 4 ml of liquid medium was added. Three days later, overlays were removed, monolayers were washed once with PBS, and cells were fixed with a mixture of acetone and ethanol (3:2) for 5 to 10 min at room temperature. REV foci were subsequently visualized by using MAb 11A25 (13) as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry) as the secondary antibody. Monolayers were washed three times with PBS following each antibody treatment. The substrate solution consisted of freshly prepared 3,3'-diaminobenzidine tetrahydrochloride (0.6 mg/ml; Eastman Kodak, Rochester, N.Y.), 0.03% $CoCl_2$, and 0.03% H_2O_2 in 45 mM Tris-HCl. Foci appeared almost immediately after the addition of substrate (4 ml per dish) and reached maximum intensity after 5 to 10 min. The titer of a serum is defined as the reciprocal of the serum dilution which gives 50% neutralization.

In some cases, neutralizing antibody titers from individual birds were estimated by using a limiting-dilution endpoint modification of the immunoperoxidase assay described above, conducted in microtiter plates. Briefly, plasma samples were diluted 1:2.5 in medium to give a total volume of 200 μl. Serial twofold dilutions were made in microtiter plates, and an equal volume (100 μl) of medium containing 20 FFU of SNV was added to each well. Neutralization was allowed to proceed for 30 min at room temperature, and then 50 μl of medium containing 5×10^4 CEF was added to each well. Microtiter plates were incubated for 7 days at 37°C, with a medium change on day 5. Medium was removed, and cells were fixed in acetone-ethanol (3:2) and allowed to dry. The fixed cells were rehydrated and assayed for SNV envelope antigen, using MAb 11A25, peroxidase-conjugated goat anti-mouse IgG, and 5-aminosalicylic acid–H_2O_2 substrate exactly as described above for ELISA assays. Wells were examined by eye (without SDS solubilization) for the presence or absence of virus growth. The neutralization titer is defined as the greatest dilution that gives complete neutralization of the virus. The medium used as diluent for plasmas, virus, and cells contained 10% calf serum. The additional serum increased the contrast between infected and uninfected wells by stimulating the rapid growth of SNV.

In addition, individual birds were weighed on the day of challenge and periodically thereafter.

RESULTS

Generation and analysis of stable FPV-SNV recombinants. Plasmid transfer vectors of the type shown in Fig. 1 were used to recombine a gene cassette, consisting of the SNV *env* gene and the *Escherichia coli lacZ* gene, into the FPV genome. FPV-infected cell cultures were transfected with plasmid as described in Materials and Methods, and the resulting recombinant FPVs formed blue plaques in the presence of Bluo-gal. The initial frequency of blue recombinant plaques ranged from about 0.01% up to nearly 2% in several experiments. Plaque purification of recombinants was continued until all plaques stained blue with Bluo-gal. This usually took four or five passages.

To test the stability of recombinants, blue plaques from the final round of plaque purification were disrupted by sonication and amplified on CEF monolayers without regard to *lacZ* phenotype. Two additional blind passages were performed, using a small amount of sonicated lysate as the inoculum for the next passage. The remainder of the lysate from each round was stored at −20°C until the conclusion of the final passage, when samples from each passage were plaqued and stained with Bluo-gal. The blue-plaque phenotype persisted after three blind passages (at least six generations of virus growth), which is consistent with a stable double-crossover-mediated recombination event into a nonessential region of the genome. A few white plaques were seen in some cases, but they did not increase in frequency in subsequent passages. These plaques probably represent point mutations or other small changes in the *lacZ* gene rather than a deletion of the *lacZ-env* cassette.

Southern hybridization analysis was performed on all eight recombinants to determine the physical structure of the viral DNA near the insertion sites of the *lacZ*-SNV *env* cassette. Three probes were used: (i) a probe to the SNV *env* gene, which showed this gene to be present in all of the recombinant viruses but not in parental FPV, (ii) a probe to plasmid sequences, which showed these sequences to be absent in both recombinant and parental viruses, and (iii) a probe to either position 29 or position 25 of the FPV genome, which showed a shift in the number and size of restriction enzyme fragments in the recombinants, relative to parental FPV. These data, taken together, clearly indicate that all recombinants possess the predicted genomic structures. Specifically, they are the result of stable insertions of the *lacZ* and SNV *env* genes into the expected positions of the FPV genome, mediated by double-crossover recombination events.

Expression of the SNV *env* gene in vitro. Indirect immunofluorescence, using either polyclonal anti-REV chicken sera or MAb 11A25, was applied to detect the expression of the SNV *env* gene in infected cells as described in Materials and Methods. Low levels of envelope glycoprotein were detected when the vaccinia $P_{7.5}$ promoter was used for expression of the *env* gene, whereas much higher levels of expression were seen when the P_s promoter was used to drive the SNV *env* gene (Table 1).

The relative expression levels of the *env* gene in the eight recombinant FPVs were quantified by ELISA (Table 1). Infection of cells with any of the four recombinant FPVs which use the $P_{7.5}$ promoter resulted in OD_{490} readings averaging between 0.299 and 0.362. The four recombinants utilizing P_s gave average OD_{490} values ranging from 1.167 to 1.280. Thus, P_s appears to be driving three- to fourfold higher levels of antigen expression than $P_{7.5}$. The insertion site (position 25 versus position 29) and the orientation (right

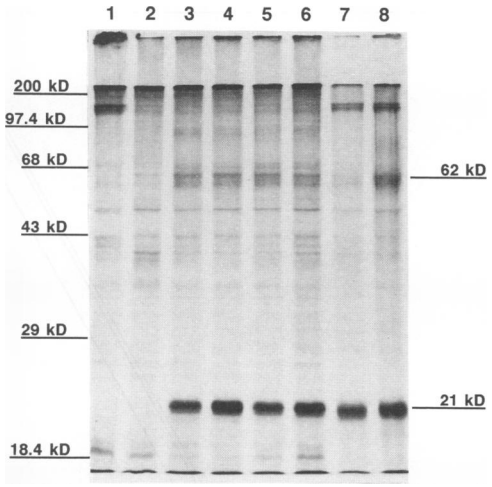

FIG. 2. Radioimmunoprecipitation of REV envelope glycoproteins. Infected cells were metabolically labeled with [^{35}S]methionine, and REV envelope glycoproteins were immunoprecipitated with MAb 11A25 as described in Materials and Methods. The following viruses were used: lane 1, mock-infected cells; lane 2, parental FPV; lane 3, f25R-SNenv; lane 4, f25L-SNenv; lane 5, f29L-SNenv; lane 6, f29R-SNenv; lane 7, SNV; lane 8, REV-T(F). The positions of ^{14}C-labeled molecular weight markers are shown on the left, and two REV *env*-related peptides are indicated on the right.

versus left) seem to have little or no influence on expression levels.

Immunoprecipitation of ^{35}S-labeled proteins revealed that all four of the P_s-based recombinant FPVs produced significant amounts of SNV envelope glycoprotein (Fig. 2). Two MAbs were used. The results with MAb 11A25 are shown. MAb 11B118 gave virtually identical results. Both of these MAbs have previously been shown to detect two peptide species, with relative molecular sizes of about 62 and 21 kDa, in REV-infected cells (13). These two peptides were detected in SNV- and REV-T-infected cells in this experiment and also in cells infected with any of the four recombinant FPVs but not in mock-infected or parental FPV-infected cells.

Protection of chickens from SNV challenge. In two separate experiments, chickens were vaccinated with FPV recombinant f29R-SNenv at 1 day of age and challenged with SNV 2 weeks later. These birds were bled at intervals and assayed for viremia and neutralizing antibody. The results are shown in Table 2. In experiment 1, 10 of 15 unvaccinated chickens and 12 of 13 birds vaccinated with parental FPV were positive for SNV viremia 1 week postchallenge. Virus titers in plasmas from viremic birds averaged 4.6×10^3 and 3.3×10^4 FFU/ml, respectively. In contrast, SNV was not detected in any of the 15 chickens which were vaccinated with recombinant f29R-SNenv. By 2 weeks postchallenge, virus had been cleared from all but one bird (this bird was negative for viremia at 3 weeks postchallenge). One of the viremic chickens in the unvaccinated group died between 1 and 2 weeks postchallenge. All of the f29R-SNenv-vaccinated birds remained viremia negative. In experiment 2, viremia was measured at 4, 8, and 12 days postchallenge. Ten of 14 unvaccinated birds had detectable levels of SNV in at least one of these time points, whereas none of the 15 vaccinated birds were viremic at any time point (Table 2).

In experiment 1, neutralizing antibodies to SNV could not be detected in pools of plasma from unvaccinated or parental FPV-vaccinated chickens at 1 week postchallenge, when most of these birds were shedding large amounts of virus into the plasma (Table 2). High titers of neutralizing antibody developed during the next week, and this finding correlates well with the observed clearing of virus in these birds. Plasma from the recombinant FPV-vaccinated group, in contrast, already contained a moderate level of neutralizing activity by 1 week postchallenge, which may account for the absence of viremia in these birds. There was no rapid increase in antibody titer between 1 and 2 weeks in these birds, as was seen in the other two groups. In experiment 2, samples were collected at day 0 (prechallenge) and at 8, 12, and 33 days after challenge. Neither the vaccinated nor the unvaccinated groups had detectable neutralizing antibody (titer of <5) prior to challenge or at 4 days following challenge. At 8 days postchallenge, however, a marked difference between the groups was observed. Unvaccinated birds still lacked neutralizing activity, whereas activity in the vaccinated group had peaked, with a neutralizing titer of 310 (Table 2 and Fig. 3). At 12 days postchallenge, the unvaccinated group reached a peak titer of 210. The neutralizing titer of plasmas from individual birds was estimated at termination (33 days postchallenge), using a rapid microtiter assay. Neutralizing titers among vaccinated birds ranged from 5 to 640, with one bird showing a titer of less than 5. The unvaccinated group had titers ranging from 40 to greater than 10,240, with two birds lacking detectable levels of neutralizing antibody. The apparent failure of 3 of the 29 birds to seroconvert suggests that the effective challenge dose may have been quite low, perhaps only 2 to 3 50% infective doses per bird.

TABLE 2. Protection of chickens vaccinated with f29R-SNenv from SNV challenge[a]

Expt	Vaccination	Viremia[b]					Neutralizing antibody[c]							
		4[d]	7	8	12	14	0	4	7	8	12	14	21	33
1	f29R-SNenv	ND[e]	0/15	ND	ND	0/15	ND	ND	50	ND	ND	90	30	ND
	Parental FPV	ND	12/13	ND	ND	0/13	ND	ND	<5	ND	ND	100	200	ND
	Unvaccinated	ND	10/15	ND	ND	1/14	ND	ND	<5	ND	ND	400	250	ND
2	f29R-SNenv	0/15	ND	0/15	0/15	ND	<5	<5	ND	310	100	ND	ND	18
	Unvaccinated	8/14	ND	4/14	2/14	ND	<5	<5	ND	<5	210	ND	ND	120

[a] Chickens were vaccinated at 1 day of age, challenged with SNV at 2 weeks, and bled for viremia and antibody periodically as described in Materials and Methods.
[b] Ratios of viremic birds to total surviving birds. The limit of detection is 2 FFU/ml of plasma.
[c] The neutralizing antibody titers of pooled plasmas were determined by using a focus reduction assay as described in Materials and Methods.
[d] Days postchallenge.
[e] ND, not determined.

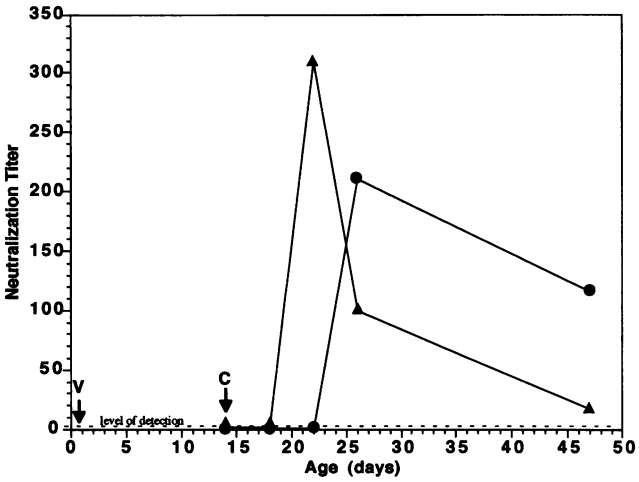

FIG. 3. Onset of neutralizing antibody activity in vaccinated and unvaccinated chickens following challenge with SNV. Birds were vaccinated (V) at 1 day of age with FPV recombinant f29R-SNenv (▲) of left unvaccinated (●). Challenge (C) with SNV was at 2 weeks of age. Neutralizing antibody titers in pooled plasmas (14 or 15 birds per group) were determined at the time of challenge and at 4-day intervals thereafter, using an immunoperoxidase-based focus reduction assay as described in Materials and Methods.

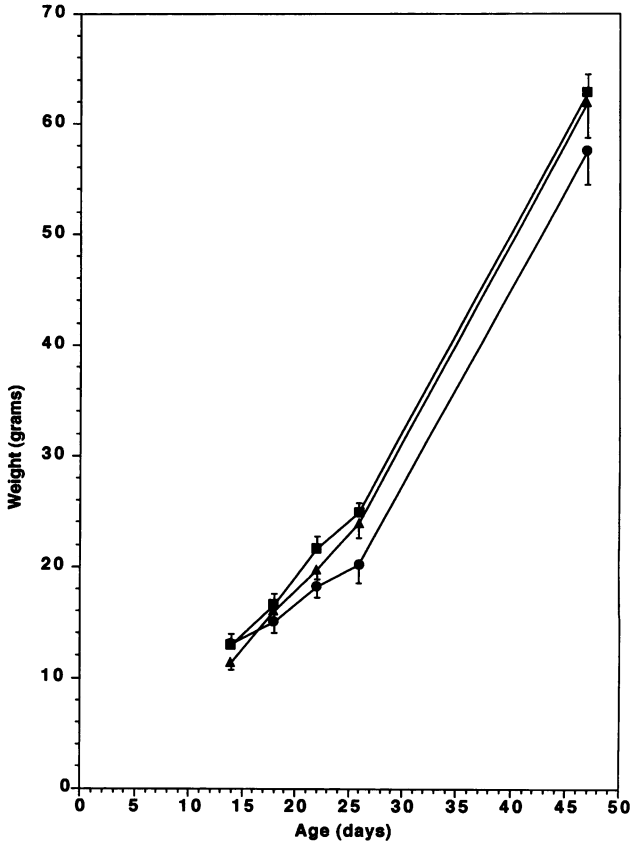

FIG. 4. Weight gain by vaccinated and unvaccinated chickens following challenge with SNV. Birds were vaccinated at 1 day of age with FPV recombinant f29R-SNenv (▲) or were left unvaccinated (●, ■). At 2 weeks of age, birds were challenged with SNV (▲, ●) or left unchallenged (■). They were weighed individually beginning at the day of challenge. The weights of female birds were multiplied by an age-dependent conversion factor, such that the overall average weight of male and female birds are equal at all time points. These factors are 1.08 at 14 days of age, 1.11 at 18 days, 1.15 at 22 days, 1.19 at 26 days, and 1.27 at 47 days. One standard deviation is indicated by the error bars.

Individual birds were weighed in experiment 2, beginning on the day of challenge (Fig. 4). An unvaccinated and unchallenged control group was included for comparison. At the time of challenge, the vaccinated group was slightly but significantly smaller than the other two groups. This difference is possibly due to adverse effects resulting from the trauma associated with handling and injection. In spite of this initial size disadvantage, the vaccinated group rapidly overtook the unvaccinated group during the first 4 days following challenge with SNV. By 12 days postchallenge, the average weight of the unvaccinated birds was more than 2 standard deviations less than the average weight of the vaccinated birds. Although the unvaccinated birds eventually resumed a normal growth rate, they never recovered from the SNV-induced runting syndrome, remaining significantly smaller than the protected group throughout the experiment (Fig. 4).

DISCUSSION

Eight recombinant FPVs were generated, all of which express the envelope glycoprotein of the SNV strain of the avian retrovirus REV. The various recombinants utilized two different nonessential insertion sites in the FPV genome, two different poxvirus promoters for *env* gene expression, and both orientations of the foreign gene relative to flanking FPV sequences. The most important determinant in the expression of the SNV envelope antigen from these recombinants was the strength of the promoter used. $P_{7.5}$ is a naturally occurring late/early promoter of moderate strength which drives expression of the 7.5-kDa polypeptide of vaccinia virus (48). P_s is a synthetic late/early promoter whose sequence is based upon extensive optimization experiments to maximize transcription from early (14) and late (15) vaccinia virus promoters. The sequence and synthesis of P_s are described elsewhere (53). The recombinants which utilized the P_s promoter expressed much higher levels of envelope glycoprotein than did the recombinants which used the $P_{7.5}$ promoter, as determined by immunofluorescence microscopy and by ELISA (Table 1).

Two nonessential insertion sites within the FPV genome have been used extensively in our laboratory for the generation of recombinant FPVs. These are designated positions 25 (32) and 29 (53). Consistent with results reported elsewhere (32), insertion of foreign DNA into position 25 results in recombinants which display a small-plaque phenotype, as a result of disruption of a gene whose product is involved in the release of enveloped virions. Despite the differences in plaque size, the choice of insertion site made no apparent difference in the amount of antigen expressed in ELISA (Table 1) or in a radioimmunoprecipitation assay (Fig. 2), and a position 25 recombinant was at least as efficient as two position 29 recombinants at eliciting neutralizing antibodies in chickens after multiple injections (data not shown).

The orientation of the inserted gene cassette into a nonessential site determines the direction of transcription of the foreign genes relative to flanking FPV genes. Conceivably, strong promoters flanking the insertion site could interfere with (or enhance) transcription of the *env* or *lacZ* gene.

Conversely, the strong P_s promoter used to drive expression of the *env* gene could influence the transcription levels of downstream FPV genes, to the detriment of the vector. These effects are possibilities despite the efforts taken to minimize them during design of the transfer vectors (orientation of the two foreign genes to give converging transcription, with separation by a bidirectional terminator of early transcription, for example). In the studies reported here, we observed no such orientation effects, either in terms of the expression levels of envelope glycoprotein or β-galactosidase or in terms of the growth rate, plaque size, and overall viability of the FPV vector.

To the extent that it can be determined from the immunoprecipitation data (Fig. 2), posttranslational modifications of the envelope glycoprotein in cells infected with the recombinant FPVs seem to be identical to those in REV-infected cells. In both systems, MAbs 11A25 and 11B118 precipitate low levels of a 62-kDa, broadly banding polypeptide which probably represents the uncleaved and underglycosylated precursor of both the surface and transmembrane peptides. In addition, higher levels of the 21-kDa mature transmembrane protein were detected in both recombinant FPV- and REV-infected cells.

The ability of these recombinant FPVs to elicit neutralizing antibodies, to block the growth of SNV in immunized chickens (Table 2), and to prevent the runting syndrome associated with SNV (Fig. 4) suggests that these viruses may be useful as vaccines. The mechanism of protection appears to involve priming of humoral immunity prior to challenge, such that neutralizing antibodies develop more rapidly in vaccinated birds (between 4 and 8 days after SNV challenge) than in unvaccinated controls (8 to 12 days postchallenge; Fig. 3). It is also possible that cell-mediated immunity contributes to the observed protection.

Chen et al. (12) compared sera from chickens infected with 26 separate isolates of REV for the ability to cross-neutralize. These viruses were originally isolated from turkeys, chickens, ducks, and pheasants as well as from contaminated vaccine and virus stocks. All isolates, including SNV, elicited antisera that were capable of neutralizing all other isolates to a significant degree, with relative neutralizing titer ratios (homologous/heterologous) ranging from 1:1 to 1:16. The implication of this study is that a vaccine which protects well against one strain of REV may protect against other strains. Consistent with this hypothesis is the finding that sera from chickens immunized with three of the SNV envelope-expressing recombinant FPVs reported here are capable of neutralizing not only SNV (Table 1) but also REV-A (data not shown).

Recombinant FPVs expressing REV genes, as described in this study, might serve as successful vaccines against REV-associated diseases. Vaccination against REV could be used either (i) to stimulate maternal antibodies in breeders, in order to provide passive protection in progeny during the first 2 to 3 weeks of life, when they are most susceptible to infection, or (ii) to directly stimulate an adequate immune response in the progeny that would protect against early exposure to REV. This second type of vaccination would likely have to be administered as a single dose at 1 day of age in order to be economically feasible to the poultry producer. The clear reduction of viremia and runting disease symptoms following challenge of vaccinated chicks predicts that this method may offer protection from REV-induced immunodepression and lymphomas, but confirmation by additional studies is required. FPV grows well in turkeys as well as in chickens, and vaccine strains of FPV have routinely been used for the vaccination of turkeys (45). The recombinant FPVs described here would therefore be expected to perform well in turkeys as well, although these tests remain to be done.

ACKNOWLEDGMENTS

We thank Barry Coulson for assisting in the in vivo testing of recombinants, as well as Barbara Riegle and Laurie Welch for performing serum neutralization assays.

These studies were funded in part by an ARS technology transfer agreement with Nippon Zeon Co., Ltd., Tokyo, Japan.

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