Field and Vaccine Strains of Fowlpox Virus Carry Integrated Sequences from the Avian Retrovirus, Reticuloendotheliosis Virus[1]

Christian Hertig,* Barbara E. H. Coupar,† Allan R. Gould,† and David B. Boyle†[,2]

*Institute of Veterinary Virology, University of Bern, Langgass-Strasse 122, 3001 Bern, Switzerland; and †CSIRO Division of Animal Health, Australian Animal Health Laboratory, Private Bag 24, Geelong, Victoria, 3213, Australia

For baculoviruses and herpesviruses, integration of transposons or retroviruses into the virus genome has been documented. We report here that field and vaccine strains of fowlpox virus (FPV) carry integrated sequences from the avian retrovirus, reticuloendotheliosis virus (REV). Using PCR and hybridization analysis we observed that vaccine and field strains of FPV carry REV sequences integrated into a previously uncharacterized region of the right 1/3 of the FPV genome. Long-range PCR, hybridization, and nucleotide sequence determination demonstrated that one vaccine strain (FPV S) and recently isolated field strains carry a near-full-length REV provirus. For another vaccine strain (FPV M) a rearranged remnant of the LTR was found at the same insertion site. By Western blotting and reverse transcriptase assays we were unable to demonstrate free REV in supernatants of FPV S cultures. The near-full-length REV provirus integrated into the FPV genome is infectious since FPV S DNA gave rise to REV upon transfection into chicken embryo fibroblasts. Upon infection of chickens with FPV S, all chickens developed high-titered antibodies to REV, and REV was isolated from the blood of half of the inoculated chickens. Our observations add to the list of targets for retrovirus integration into DNA virus genomes. The integration of a near-full-length, and apparently infectious, REV provirus into FPV provides additional transmission routes for the retrovirus by way of the infectious cycle of FPV, including the possibility of mechanical transmission by biting insects since FPV is believed to be transmitted by this route. For large DNA viruses, including the poxviruses, retrovirus integration with attendant possibilities of gene transduction may be an important mechanism for virus evolution, including the acquisition of cellular genes with the potential to modify virus virulence and pathogenicity. © 1997 Academic Press

INTRODUCTION

Among the large DNA viruses, baculoviruses have been identified as being able to spontaneously accommodate host cell-derived transposons (Blissard and Rohrmann, 1990). A number of different mobile genetic elements from lepidopterans have been identified as inserts within the large (~128 kb) DNA genome of *Autographa californica* nuclear polyhedrosis virus (the prototype of baculoviruses subgroup A). These mobile genetic element inserts range in size from a few hundred basepairs to 7.5 kb of the retrotransposon TED, which encodes *gag* and *pol* proteins that assemble into virus-like particles containing reverse transcriptase activity (Lerch and Friesen, 1992).

In a parallel observation reticuloendotheliosis virus (REV) was shown to have integrated into the genome of an avian herpesvirus, Marek's disease virus (MDV), during mixed infections of cell cultures used for attenuation of the MDV by continuous passage (Isfort *et al.*, 1992). Although insertion sites appeared clustered, insertions

did occur in several different regions of the genome (Jones *et al.*, 1993). Integrated proviruses were unstable in undergoing recombination deletion, leaving fragments of the long terminal repeats in the MDV genome. Coinfection studies with two different avian retroviruses, REV and avian leukosis virus (ALV), and two different avian herpes viruses, MDV and the herpesvirus of turkeys (HVT), showed that integration of retrovirus into the herpesvirus genome could occur as early as the fourth to sixth *in vitro* passage (Isfort *et al.*, 1994). Integration occurred at a number of sites including the HVT gD gene which was disrupted by the insertion. In one case the integrated REV provirus in the HVT genome appeared to be full length as it was infectious when transfected into chicken embryo fibroblast cells (Isfort *et al.*, 1994). Short regions of nucleotide sequence homology within the R and U3 regions of REV LTR observed in other isolates of MDV type I suggest that REV insertion into the MDV genome has occurred frequently in the past (Isfort *et al.*, 1992). To date no field isolates of MDV have been described containing full-length REV provirus. Retrovirus integration into the genome of herpesviruses has the potential to generate all the changes, e.g., gene activation, mutation, that are usually associated with retrovirus integration into cellular genomes, as well as providing a novel means of retrovirus transmission via the infectious

---

[2] To whom correspondence and reprint requests should be addressed. Fax: +61 3 5227 5555. E-mail: david.boyle@aahl.dah.csiro.au.

cycle of the herpesvirus. In one MDV isolate, insertional activation of MDV genes by the integrated REV LTR promoter has been demonstrated. This isolate also has a modified *in vivo* phenotype, although a direct relationship between the insertional activation of genes and this phenotype has not been established (Jones *et al.,* 1996). Thus retrovirus integration into herpesvirus genomes may have important influences on the evolution of these viruses.

For both baculoviruses and herpesviruses, DNA replication occurs within the nucleus of an infected cell. Integration of the provirus into the cellular DNA occurs providing an opportunity for viral genomes to be an alternative target for integration. In contrast, poxvirus DNA replication occurs within the cytoplasm of the infected cell. Nonetheless REV appears able to integrate into the genome of FPV as we present evidence here that vaccine and field strains of FPV carry integrated REV sequences. Some of these FPV strains carry a near-full-length provirus of REV. Although this REV provirus has an incomplete 3′ LTR, our data show that it can give rise to infectious REV when FPV S DNA is transfected into cell cultures and when chickens are infected with FPV S. This is the first evidence for natural retroviral integration into a poxvirus genome. It provides a novel means for transmission of the retrovirus via the infectious cycle of the carrier poxvirus and may influence the evolution of both viruses. The potential for the acquisition of cellular genes via the integration of a retrovirus into a poxvirus genome is consistent with the evidence for a wide range of cellular gene homologues in poxvirus genomes, particularly genes whose functions modify the host immune response to the poxvirus.

MATERIALS AND METHODS

Cells, viruses and virus DNA

Australian FPV vaccines, FPV M (mild vaccine strain) and FPV S (standard vaccine strain), were obtained from Cyanamid-Websters Pty. Ltd., Castle Hill, Australia. The FPV S vaccine is not currently used in Australia — its use was discontinued because of suspected REV contamination. The FPV M has been widely used in Australia and is known to be free of REV. Plaque-purified derivatives of this strain (designated FPV M3 and FPV M A to F) have been described elsewhere (Boyle *et al.,* 1997). Field isolates of FPV, designated AWPL 1136, 1137, 1138, 1139, and 1140, were obtained from scab material collected from poultry infections occurring in New South Wales, Australia during 1988 and 1989. A comparison of these field isolates with the vaccine strains has been described elsewhere (Boyle *et al.,* 1997). An Australian isolate of REV, designated REV/Vic/1/76, was obtained from Dr. J. Ignatovic (CSIRO, Parkville, Australia) and cultivated in chicken embryo fibroblast (CEF) cell cultures (Bagust and Dennett, 1977).

Primary chicken embryo skin cell cultures (CES) were prepared from 13-day-old specific pathogen-free (SPF) embryos (Silim *et al.,* 1982). Chicken embryo fibroblast (CEF) cell cultures were prepared from 10-day-old SPF embryos. FPV DNA was extracted from partially purified FPV grown in CES cells and DNA restriction endonuclease fragments were separated by horizontal agarose gel electrophoresis, transferred to hybridization membranes, and hybridized with radioisotope-labeled probes (Coupar *et al.,* 1990).

REV LTR PCR

The primers and protocols described by Aly *et al.* (1993) were used for amplification of REV LTR sequences present in FPV S, the AWPL field strains, and CEF cells infected with REV virus. ^{32}P-labeled REV LTR PCR products were prepared by including ^{32}P-labeled nucleotide in the PCR mix with a corresponding reduction in the equivalent unlabeled nucleotide to 1/10 the normal concentration.

XL PCR analysis of REV sequences in FPV

XL PCR (Perkin–Elmer GenAmp XL PCR) was used to characterize the REV sequences in FPV S strain. Primer 1 (sense) (5′-CCATCGAATTCACGTATTAC-3′) located at the *Eco*RI end of the sequenced region of FPV M3; primer 2 (antisense) (5′-CGGAATTCGGATCCGCGTGAATAGCT-TCTACGGG-3′) located at the *Bam*HI end of the FPV M3 sequenced region (*Eco*RI site added to aid cloning); primer 3 (sense) (5′-TTTCTGCATCCCTCTGGC-3′) derived from the polymerase region of REV (sequence determined from the *Eco*RI–*Pst*I fragment of FPV S); and primer 4 (antisense) (5′-CGAGCCAGAGACCTAGTAGC-3′) derived from the end of the polymerase region of REV (Ref ACRPOLENV, K02537; GenBank). PCR conditions included the use of a hot start, 93° for 1 min, 55° for 2 min, 68° for 5 min with a 10-sec extension per cycle using a total of 30 cycles. Less than 100 ng of FPV DNA was used as template in each 100-μl reaction.

Restriction enzyme mapping, cloning, and nucleotide sequence determination of REV insertion site

FPV M3 and S genomic DNA fragments carrying REV sequences were separated by agarose gel electrophoresis after restriction endonuclease digestion. Selected DNA bands were excised from the gel, purified, and cloned into the pUC19 plasmid vector. Desired plasmids were identified by hybridization and restriction endonuclease digestion analysis. Initial nucleotide sequence was determined from these plasmids using universal forward and reverse sequencing primers. Additional sequence was obtained by a variety of strategies including cloning of subfragments into pUC19 and M13 vectors and primer walking strategies, followed by manual and automated sequencing methods. The location of the REV

LTR insertion within the FPV genome was mapped by hybridization of selected fragments to restriction endonuclease-digested genomic DNA. The previously constructed PstI and partial BamHI maps of the FPV genome (Coupar et al., 1990) were used to locate the REV LTR insertion within the FPV genomic map.

Reverse transcriptase assay and Western blotting

Supernatants from FPV S- and FPV M3-infected CES cell cultures and from REV-infected CEF cell cultures were clarified by centrifugation at 2500 rpm for 10 min. Half of the resulting supernatants were directly centrifuged at 28,000 rpm in a Beckman SW28 rotor for 3 hr at 4° and the second half was similarly centrifuged after ultrafiltration through 0.2-μm filters to remove remaining cell debris and FPVs. A greater than 100-fold concentration of the culture supernatants was achieved by the ultracentrifugation. Pellets were resuspended in RT lysis buffer (30 mM Tris–HCl, pH 8.0, 80 mM KCl, 1.0 mM EDTA, 0.1% v/v Triton X-100, 10% v/v glycerol, and 2 mM DTT) and dilution series were tested for reverse transcriptase activity using RT-detect (NEN DuPont) according to the manufacturer's instructions. The 4× RT buffer used contained 130 mM Tris–HCl, pH 8.0, 120 mM KCl, and 33.6 mM $MgCl_2$. For immunoblot analysis equal amounts of 2× SDS gel-loading buffer (100 mM Tris–HCl, pH 6.8, 20% v/v glycerol, 4% w/v SDS, 0.2% w/v bromophenol blue, 200 mM DTT) and pelleted material resuspended in RT lysis buffer were mixed. The samples were resolved by SDS–PAGE on 12% gels and transferred onto nitrocellulose membranes by electroblotting. After the membranes were blocked with 5% nonfat powdered milk in 0.02% Tween 20 and PBS, they were incubated with a monoclonal antibody to the gag (P29) product of REV for 1 hr, washed three times, incubated with sheep anti-mouse antibody conjugated with horseradish peroxidase (Silenus), washed three times, and reacted with 4-chloro-1-naphtol and hydrogen peroxide.

In vitro recovery of REV from FPV S DNA

Cellular genomic DNA was prepared from uninfected and REV-infected CES and CEF cell cultures. DNA was prepared from 5 × 10^6 cells using the Qiagen genomic DNA purification procedure (Qiagen Pty. Ltd.). Cultures infected with REV were inoculated as 50–80% confluent monolayers and cells were harvested for DNA extraction 10 to 14 days later.

FPV DNA was purified from FPV S- and M3-infected CES cell cultures. CES cell cultures were infected at a multiplicity of 0.05 to 0.1 PFU per cell. When the CPE had reached 80 to 90% (5 to 8 days after infection) the cells were harvested, resuspended in 4° 10mM Tris–HCl, pH 7.6 (20 ml per 5 × 10^7 cells), and dounce homogenized. Nuclei were removed by centrifugation at 5000 g for 5 min and RNase (20 μg/ml) and DNase (25 μg/ml)

added. After incubation for 15 min at 37°, trypsin was added to a final concentration of 250 μg/ml followed by a further incubation for 15 min at 37°. Twenty milliliters of extract was layered onto a 16-ml cushion consisting of 36% sucrose (8 ml) overlaid with 10% dextran T10 (8 ml) in 10 mM Tris–HCl, pH 7.6. Virus was pelleted by centrifugation at 20,000 rpm for 80 min in a SW28 Beckman rotor and then resuspended in 18 ml 10 mM Tris–HCl, pH 7.6, 1% Triton X-100, and 35 mM mercaptoethanol. Following a further 10-min incubation on ice with occasional gentle mixing, viral cores were pelleted by another round of ultracentrifugation through the dextran/sucrose cushions. DNA was extracted from the cores using the Qiagen genomic DNA purification procedure.

As a control to demonstrate the removal of REV cellular provirus DNA and free REV during purification of FPV DNA, FPV M3-infected CES cells were mixed with an equal number of REV-infected CES cells prior to commencing the protocol for purification of FPV DNA.

Purified cellular and FPV DNAs (0.5–1 μg) were digested to completion with EcoRI [the REV provirus does not contain any EcoRI sites (Chen et al., 1981)]. The DNA was then transfected into 50–80% confluent monolayers of CEF cells using Lipofectamine (Gibco BRL). Ten to fourteen days later the culture supernatants were harvested and passaged onto fresh 50–80% confluent monolayers of CEF cells. Supernatants from the second passage were harvested 10 days later. CEF cells were infected in chamber well slide cultures with both the first- and the second-passage culture supernatants from the transfections. Ten days later the monolayers were fixed with methanol and stained by immunofluorescence with the monoclonal antibody to the REV gag (p29) gene product.

In vivo recovery of REV from FPV S

Fifteen chickens at 3 weeks of age (SPF hybrid white leghorn strain) were inoculated by wing web stab and subcutaneous injection into the wing web (0.05 ml/chicken). To ensure isolation from possible sources of REV infection the chickens were held in a PC3 animal containment facility isolated from all other poultry and totally protected from insect vectors. Each chicken received 2.5 × 10^6 PFU of FPV S. Prior to infection and 32 days after infection heparinized blood was collected from the wing vein. Plasma was collected for antibody assays. Antibody responses to REV were determined using a commercially available test kit (IDEXX) which is based upon a detergent- and heat-inactivated antigen preparation from the Cook strain of REV. REV was isolated from heparinized blood by direct inoculation of CEF cell cultures. After an additional passage in CEF cells, the cultures were stained by immunofluorescence with the gag (P29) monoclonal antibody to detect REV.
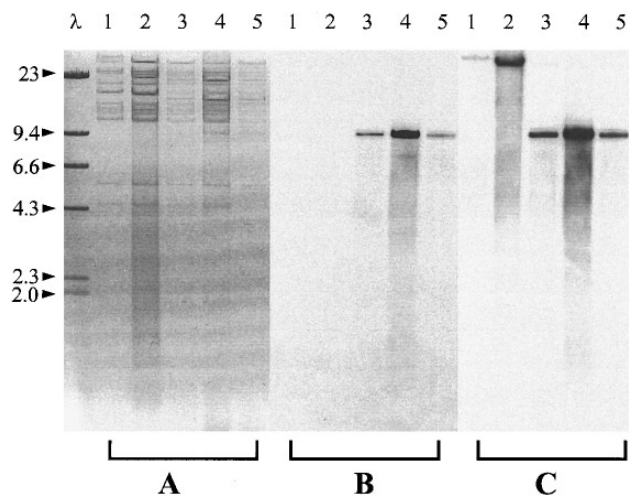
FIG. 1. Evidence for REV sequences in field and vaccine strains of FPV. FPV DNAs were digested with *Pst*I restriction endonuclease and transferred to nylon membranes by Southern blotting. (A) Ethidium bromide stained. (B) ^{32}P-labeled 291-bp LTR PCR product hybridized to the DNA fragments and autoradiographed. (C) ^{32}P-labeled cloned 9.8-kb fragment from FPV S. Lanes 1 to 5 contain DNA isolated from FPV M-F, M3, S, AWPL 1136 and 1137, respectively. Lambda *Hind*III markers are shown as size markers at the left of the figure.

RESULTS

Evidence for REV sequences in FPV S and field isolates

In checking a number of field isolates by PCR for REV contamination, we observed that a specific 291-bp product from the LTR was obtained when partially purified FPV DNA was used as the template for the PCR. Contamination by REV proviral DNA from the infected cells could not be excluded; however, the FPV S and field isolates had been cultivated on CES cells derived from SPF embryonated eggs known to be free of REV. The FPV M strain had also been cultivated on these cells and this virus was negative by PCR for REV LTR sequences.

Southern hybridization with a ^{32}P-labeled 291-bp LTR fragment generated by PCR on FPV S DNA or on DNA extracted from CEF cells infected with REV demonstrated specific hybridization to a 9.8-kb *Pst*I fragment of FPV S DNA and to a fragment of the same size in field isolates AWPL 1136 to 1140 (Fig. 1B). Hybridization to *Pst*I fragments of the FPV M, M3, or A-F DNA was not obvious; however, very weak hybridization to the largest *Pst*I fragments of these viruses was sometimes observed. The 9.8-kb fragment is absent from *Pst*I digests of FPV M and its plaque-purified derivatives, but is present in all of the AWPL series field isolates and the FPV S vaccine strain (Fig. 1A). These data suggested that part of the REV genome was present within the genome of the FPV S and AWPL isolates.

When the ^{32}P-labeled REV 291-bp PCR product was hybridized to *Eco*RI-digested DNA from FPV S and M3, strong hybridization was detected with a 17-kb fragment

from FPV S and weak hybridization with a 9-kb fragment from FPV M3 (Fig. 2). Since REV provirus is reported not to contain any *Eco*RI sites (Chen *et al.,* 1981), the difference in size of the *Eco*RI fragments from FPV S and M3 might be attributable to the insertion of a complete REV provirus (≈8.3 kb) (Chen *et al.,* 1981). The lack of a specific LTR PCR product and weak hybridization to FPV M3 was suggestive of a remnant of the LTR being present.

Cloning and mapping of REV sequences in the FPV genomes

The 9.8-kb *Pst*I fragment of FPV S, shown to hybridize with the REV LTR PCR product, was cloned into pUC. This cloned fragment hybridized to the 9.8-kb fragment present in the field isolates (Fig. 1C). Confirmation that part of this fragment was derived from FPV was shown by its hybridization to the largest *Pst*I fragments of FPV M-F and M3 (Fig. 1C). The location of the REV LTR sequences within this cloned fragment was determined by restriction endonuclease digestion and Southern hybridization. An *Eco*RI–*Pst*I subfragment (4.3 kb) was identified as containing the REV LTR hybridizing region. Nucleotide sequence determination of this fragment revealed 903 bp of apparent FPV genome sequence (adjacent to the *Eco*RI site), a complete REV LTR, and sequence of the REV *gag* region extending to the *Pst*I site—a total of 3388 bp of REV genomic sequence. The presence of REV genomic sequences up to the *Pst*I site suggested that the REV integrated sequences extended into an adjoining *Pst*I fragment.

The FPV M3 *Eco*RI genomic fragment (9.0 kb) shown to weakly hybridize to the REV LTR product was cloned into pUC19. This fragment hybridized only to the largest
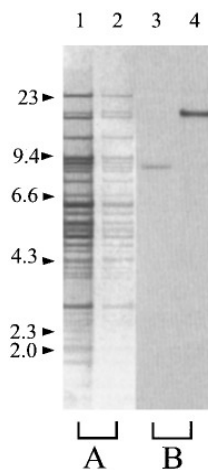
FIG. 2. Evidence for complete REV provirus in FPV S and possible remnants in FPV M3. FPV DNAs were digested with *Eco*RI restriction endonuclease and transferred to nylon membranes by Southern blotting. (A) Ethidium bromide stained. (B) ^{32}P-labeled 291-bp LTR PCR product hybridized to the DNA fragments and autoradiographed. Lanes 1 and 2 contain DNA isolated from FPV M3 and S, respectively. Lambda *Hind*III markers are shown as size markers at the left of the figure.
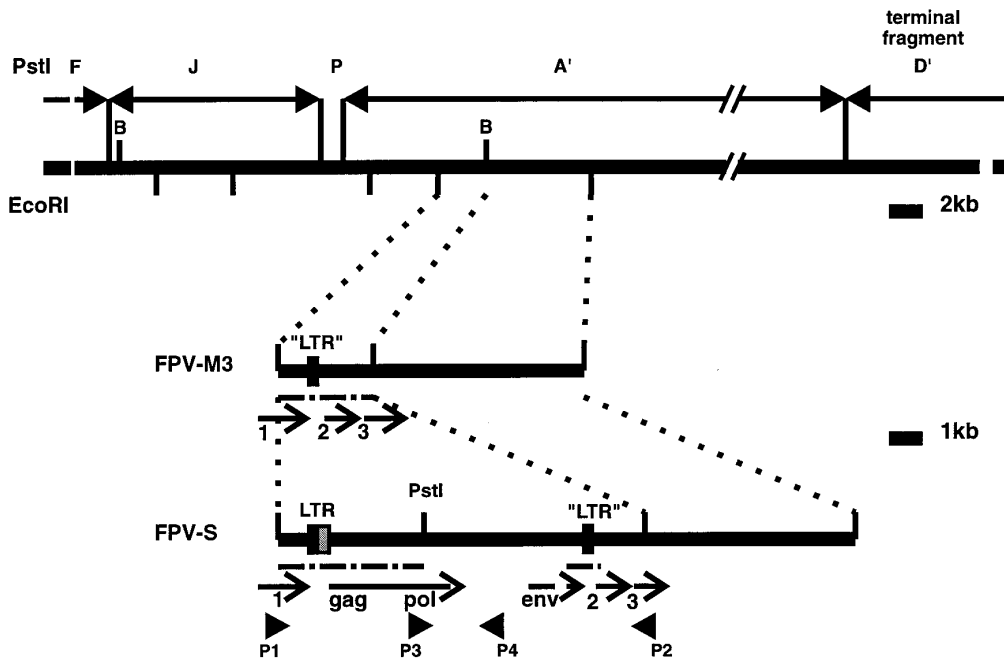
FIG. 3. Restriction endonuclease map of the FPV genome showing region of inserted REV sequences. P, *Pst*I; E, *Eco*RI; B, *Bam*HI (*Bam*HI and *Eco*RI sites marked are incomplete). LTR, long terminal repeat of REV. "LTR," truncated LTR present in FPV M3 and at the 3′ end of the REV provirus inserted into FPV S. REV genes *gag* and *env* are marked. Open reading frames from the FPV genomic region flanking the provirus insertion are marked 1, 2, and 3. *Pst*I fragments F, J, P, A′, and D′ (terminal fragment) are at the right-hand end of the FPV genome, respectively (as reported by Coupar *et al.,* 1990). Regions from which partial or complete nucleotide sequence has been determined are marked by dashed lines.

*Pst*I fragment of FPV M-F and M3, to the largest fragment of FPV S and the field strains, and to the 9.8-kb fragment of these strains shown to contain the REV LTR sequences (data not shown). Although weakly hybridizing with the REV LTR probe (Fig. 2), a specific PCR product could not be generated from FPV M3 DNA, suggesting that an incomplete REV LTR may be present in the genome of this virus. Additional restriction endonuclease mapping and nucleotide sequence determination showed that an *Eco*RI–*Bam*HI subfragment (2.8 kb) was identical to the region identified in the FPV S cloned fragment (except for the extent of REV sequences present) and appeared to span the site of insertion of the REV provirus sequences. In addition, nucleotide sequence determination revealed a truncated REV LTR (248 bp) remnant in the FPV M3 genome at the same location as the FPV S REV insertion. From these data it was possible to conclude that the REV sequences were inserted into the right-hand one-third of the FPV genome and that the REV sequences in FPV M3 and S appeared to be located at the same place within a previously uncharacterized region of the FPV genome (Fig. 3).

Although the complete sequence of the REV insert in the FPV S strain has not been determined, XL PCR analysis suggested that there was a near-full-length provirus present. XL PCR analysis of FPV M3 and FPV S using primers 1 and 2 (Fig. 3) yielded products of 2.8 kb from both virus DNAs (Fig. 4). The absence of a larger product from FPV S DNA was indicative of heterogeneity present

in the genomes of the viruses carrying near-full-length REV provirus inserts. Genome heterogeneity in these viruses would be expected since the REV LTR direct repeats present would make the genome inherently unstable in the region of the insert. This heterogeneity was not apparent in the hybridization analysis of the FPV DNAs. However, given the large size difference (2.5 kb vs ∼10 kb) between the shorter PCR product and that anticipated
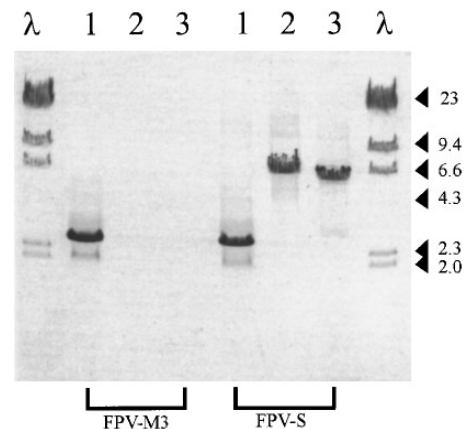
FIG. 4. Long-range (XL) PCR analysis of the REV provirus insert in FPV S strain. Primer pairs used for XL PCR were derived from lane 1 flanking FPV sequences (primer pair 1/2), from lanes 2 and 3 flanking FPV sequences and internal REV sequences (lane 2 primers 1/4 and lane 3 primers 2/3). The locations of the primers are marked in Fig. 3. λ DNA digested with *Hin*dIII was used as size markers. 10 μl from a 100-μl XL PCR was analyzed on a 0.6% agarose gel.

```
                                                  10        20
                                                   *         *

                                        | U3 →
ACRLTR1                                 TGTGGGAGGGAGCTCCGGGGGA----
                                  ← FPV |
FPV S 5'LTR    aacggaacagtaacaatataaaaag...-.................G----
FPV S 3'LTR              (REV sequence)....................A.TGTG
FPV M3 LTR     aacggaacagtaacaatataaaaag...-.................G----

                                        30        40        50
                                         *         *         *
ACRLTR1        ------------------AATAGCGCTGGCTCGCTAACTGCCATATTAGC
FPV S 5'       ------------------.............................
FPV S 3'       GGAGGGAGCTCCGGGGGG.............................
FPV M3 LTR     ------------------.............................

                        60        70        80        90       100
                         *         *         *         *         *
ACRLTR1        TTCTGTAATCATGCTTGCTTGCCTTAGCCGCCATTGTACTTGATATATTT
FPV S 5'       ..................................................
FPV S 3'       ..................................................
FPV M3 LTR     ..................................................

                       110       120       130       140       150
                         *         *         *         *         *
ACRLTR1        CGCTGATATCATTTCTCGGAATCGGCATCAAGAGCAGGCTCATAAACCAT
FPV S 5'       ..................................................
FPV S 3'       ..................................................
FPV M3 LTR     ..................................................

                       160       170       180       190       200
                         *         *         *         *         *
ACRLTR1        AAAAGGAAATGTTTGTTGAAGGCAAGCATCAGACCACTTGCACCATCCAA
FPV S 5'       ..................................................
FPV S 3'       .....A......................................-------
FPV M3 LTR     ............................................-------

                       210       220       230       240       250
                         *         *         *         *         *
ACRLTR1        TCACGAACAAACACGAGATCGAACTATCATACTGAGCCAATGGTTGTAAA
FPV S 5'       ..................................................
FPV S 3'       --------------------------------------------------
FPV M3 LTR     --------------------------------------------------

                       260       270       280       290       300
                         *         *         *         *         *
ACRLTR1        GGGCAGATGCTATCCTCCAATGAGGGAAAATGTCATGCAACATCCTGTAA
FPV S 5'       ..................................................
FPV S 3'       --------------------------------------------------
FPV M3 LTR     --------------------------------------------------
```
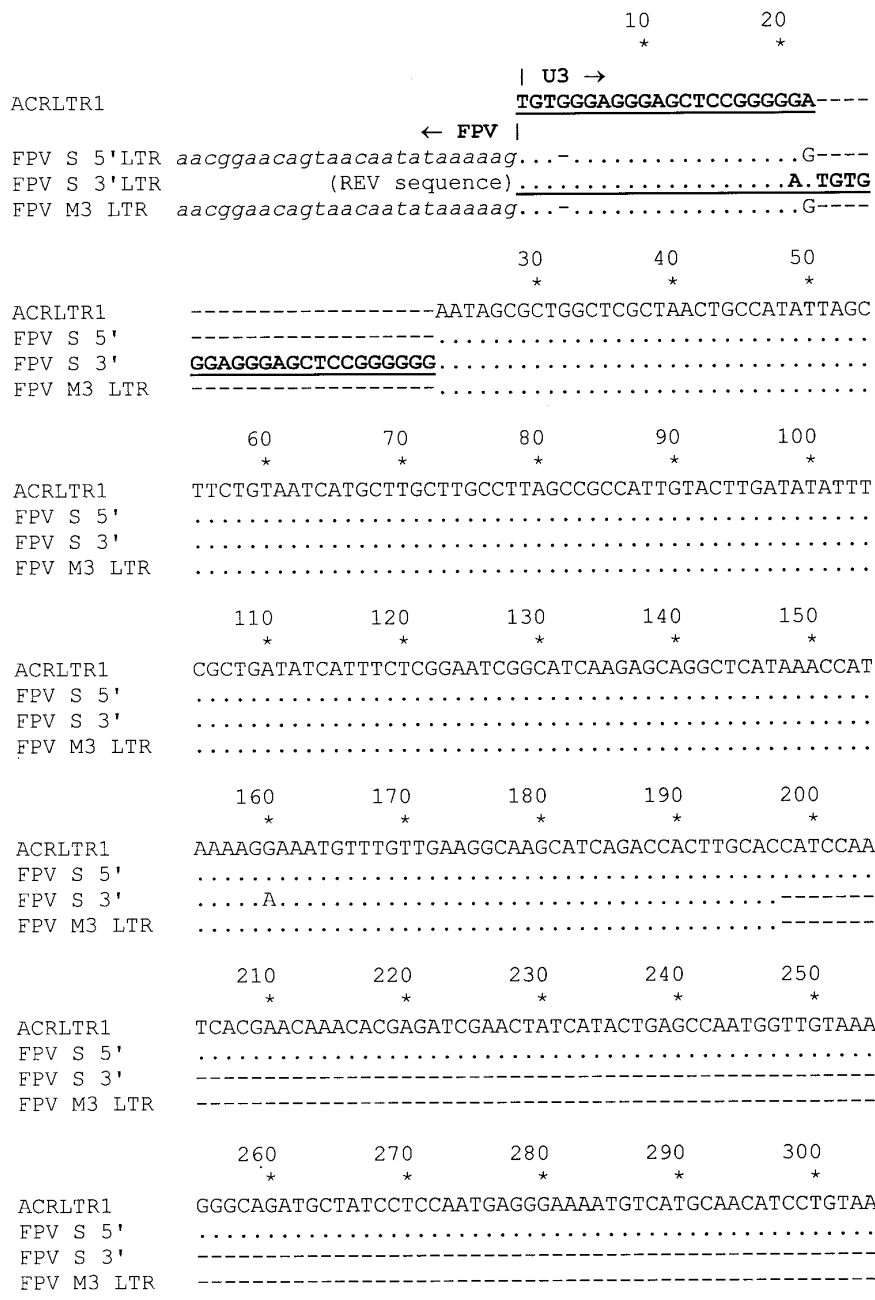
FIG. 5. Alignment of REV LTR and flanking FPV sequences present in FPV strains. The REV LTR present in chicken syncytial virus provirus (ACRLTR1) (Swift *et al.,* 1987) was aligned with REV LTR sequences present at the 5′ end (FPV S 5′) and the 3′ end (FPV S 3′) of the near-full-length provirus present in the genome of FPV S and with the REV LTR remnant present in FPV M3 (FPV M3 LTR). The U3, R, and U5 regions are those identified by Swift *et al.* (1987). Conserved nucleotides are indicated by dots; deletions are indicated by a dash. Flanking FPV sequences are shown in lowercase letters. The duplicated U3 terminus present in FPV S 3′ LTR is in boldface type and underlined. For clarity, flanking REV sequences present on the 5′ end of the FPV S 3′ LTR and the 3′ end of the FPV S 5′ LTR have been omitted.

from the near-full-length REV provirus insert, a low level of contamination with the short rearranged genome would ensure that the shorter PCR product predominated when the flanking FPV primers were used in the XL PCR. When the FPV primers were used in combination with primers derived from REV (primer pair 1 and 4) and (primer pair 2 and 3), no products were generated with FPV M3 DNA (Fig. 4). In comparison FPV S DNA gener-

ated PCR products of 5.9 and 6.3 kb, indicative of a complete REV provirus present in the FPV S DNA (Fig. 4). The 3′ end of the REV provirus inserted into FPV S was sequenced from the XL PCR product derived using primers 2 and 3. This revealed a truncated and rearranged 3′ LTR almost identical to the remnant in FPV M3 and downstream of the env gene and flanked by the same FPV genomic sequences present in FPV M3.
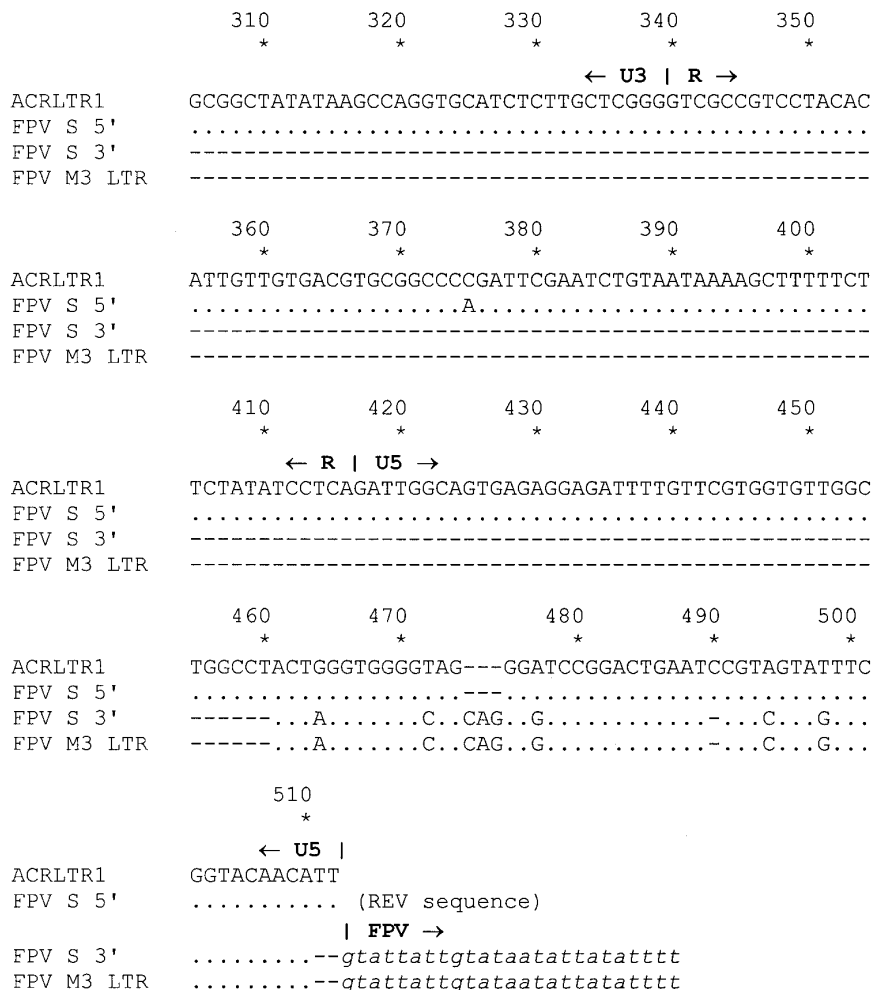
```
              310          320          330          340          350
               *            *            *            *            *
                                              ←  U3 | R  →
ACRLTR1     GCGGCTATATAAGCCAGGTGCATCTCTTGCTCGGGGTCGCCGTCCTACAC
FPV S 5'    .................................................
FPV S 3'    -------------------------------------------------
FPV M3 LTR  -------------------------------------------------

              360          370          380          390          400
               *            *            *            *            *
ACRLTR1     ATTGTTGTGACGTGCGGCCCCGATTCGAATCTGTAATAAAAGCTTTTTCT
FPV S 5'    .......................A.........................
FPV S 3'    -------------------------------------------------
FPV M3 LTR  -------------------------------------------------

              410          420          430          440          450
               *            *            *            *            *
                      ←  R | U5  →
ACRLTR1     TCTATATCCTCAGATTGGCAGTGAGAGGAGATTTTGTTCGTGGTGTTGGC
FPV S 5'    .................................................
FPV S 3'    -------------------------------------------------
FPV M3 LTR  -------------------------------------------------

              460          470          480          490          500
               *            *            *            *            *
ACRLTR1     TGGCCTACTGGGTGGGGTAG---GGATCCGGACTGAATCCGTAGTATTTC
FPV S 5'    .....................---..........................
FPV S 3'    ------...A.......C..CAG..G.............-...C...G...
FPV M3 LTR  ------...A.......C..CAG..G.............-...C...G...

              510
               *
                    ←  U5 |
ACRLTR1     GGTACAACATT
FPV S 5'    ...........  (REV sequence)
                          | FPV →
FPV S 3'    .........--gtattattgtataatattatatttt
FPV M3 LTR  .........--gtattattgtataatattatatttt
```

FIG. 5—*Continued*

The FPV genome region in which the REV near-full-length provirus integration occurred has not previously been characterized. Comparisons of the deduced proteins encoded by open reading frames (ORFs) 1, 2, and 3 (Fig. 4) with available database sequences using BLAST WWW Server (National Center for Biotechnology Information) identified distant but definite relationships with characterized genes of other poxviruses. Regions of the deduced protein (283 amino acids) encoded by ORF 1 have identifiable but very limited relationships to the molluscum contagiosum virus subtype 1 MC14 14R hypothetical protein (GenBank entry U60315) and to the A49L protein of variola virus. The protein (285 amino acids) encoded by ORF 2 is related to the hypothetical 33.6K protein (a member of the protein kinase family—287 amino acids) of Shope fibroma virus (GenBank entry JQ1743). The deduced amino acid sequence from the incompletely sequenced ORF 3 is related to the serpin from ectromelia virus (GenBank entry S24676).

The sequences of the integrated retroviral provirus are closely related to REV and spleen necrosis virus (SNV) (Fig. 5). Alignment of the FPV S 5′ LTR with REV LTR

(GenBank entry ACRLTRI) revealed a single base deletion and two base substitutions in the FPV S 5′ LTR over the 517-bp LTR region. The FPV S 3′ LTR and the remnant LTR in FPV M 3 were identical to each other except for one deletion, one base substitution, and the 23-bp duplication of the U3 5′ terminus present in FPV S 3′ LTR. However, in comparison with the FPV S 5′ LTR and the REV LTR, both the FPV S 3′ LTR and the FPV M3 remnant LTR had a large deletion (262 bp) spanning part of the U3, all of the R, and part of the U5 regions. In addition, significant changes had occurred in the remnant (51 bp) of the U5 region with three base deletions, five base substitutions, and a three-base insertion (Fig. 5). The truncated and rearranged 3′ LTR and the absence of the classical direct repeats at the integration site suggest that the integration of the REV provirus into the genome of FPV occurred in an unusual manner or underwent rearrangement after integration. The close relationship of the FPV S near-full-length REV insert to REV was further apparent upon comparison of the nucleotide and deduced amino acid sequences from the regions of REV sequence determined. Homologies ranging from 85 to

TABLE 1

Recovery of REV by Transfection of CEF Cell Cultures
with Purified DNAs

	Recovery of REV detected by immunofluorescence	
DNA purified from	First passage[a]	Second passage[a]
CES	Negative	Negative
CEF	Negative	Negative
CES (REV)[b]	Positive	Positive
CEF (REV)[b]	Positive	Positive
FPV M3	Negative	Negative
FPV M3 (REV)[b]	Negative	Negative
FPV S	Positive	Positive

[a] Purified cellular and FPV DNAs were digested with EcoRI and transfected into CEF cell cultures. 10–14 days later the supernatants were passaged onto CEF cell cultures and then the first- and second-passage supernatants were tested for REV by inoculation of CEF cell cultures in chamber well slides and staining for REV 10 days later using a monoclonal antibody to the REV gag (p29) gene product.

[b] (REV) cell cultures infected with REV- or FPV M3-infected cell cultures mixed with REV-infected CES cell cultures prior to purification of DNA.

99% were observed when compared with available REV and SNV GenBank sequences at both the nucleotide and the protein levels.

Testing biological activity of REV provirus in FPV S *in vitro* and *in vivo*

The presence of a near-full-length REV provirus integrated into FPV S posed a number of questions regarding biological function, i.e., is the apparent contamination of the FPV S due to free REV or due to REV arising from the integrated provirus? We tested for the presence of free REV virus in FPV S stocks by harvesting CES culture supernatants, concentrating any possible REV by ultracentrifugation and testing the pellet for reverse transcriptase activity and for REV *gag* antigens by Western blotting using a monoclonal antibody for antigen detection. Culture supernatants from FPV S and M (concentrated by a factor of 100-fold) were negative for RT activity and antigen by Western blotting while controls from REV-infected CEF cultures were positive. Additionally, immunofluorescence staining of CEF and CES cultures infected with FPV M, M3, S, and AWPL1136 to 1140 for REV *gag* gene products using the monoclonal antibody to *gag* (p29) was negative.

When purified and *Eco*RI-digested FPV S DNA was transfected into CEF cell cultures REV virus was recovered (Table 1). Cellular DNA purified from CEF and CES cells infected with REV yielded REV when transfected into CEF cells. DNA purified from FPV M3, uninfected CEF, and CES cells failed to yield REV upon transfection into CEF cell cultures. When DNA was purified from FPV M3 deliberately contaminated with REV-infected cells, purification of the FPV M3 DNA removed REV cellular proviral DNA and REV since REV was not detected when the DNA was transfected into CEF cell cultures (Table 1).

That FPV S could give rise to REV upon replication in chickens was determined by infecting fifteen 3-week-old SPF chickens with FPV S via the wing web. Serum samples were collected prior to infection and 32 days after infection. All 15 inoculated chickens developed high-titered antibodies to REV (10,000 to 30,000). None were positive prior to inoculation. Peripheral blood collected 32 days after infection with FPV S yielded REV from 8 of 14 chickens. This confirms the experience of using FPV S vaccine in commercial poultry where its use was associated with the apparent spread of REV. The widely used Australian FPV M vaccine and its plaque-purified derivatives are free of REV contamination when similarly tested by poultry inoculation.

DISCUSSION

Our data show that field and vaccine strains of fowlpox virus carry integrated sequences from the avian retrovirus, reticuloendotheliosis virus. PCR for REV LTR sequences identified possible REV sequences in the vaccine strain FPV S and field isolates. Hybridization analysis showed that the REV sequences are located in a previously uncharacterized region of the right-hand one-third of the genome. This also showed the possibility of REV LTR remnants in the genome of a vaccine strain known not to be contaminated with infectious REV, with the integration site being the same as for the FPV S vaccine and field strains. From restriction endonuclease analysis of the genomes and XL PCR, the data showed an almost complete REV provirus within the FPV S and field strains. Nucleotide sequence analysis of the insertion region has confirmed the conclusion that both the vaccine and the field strains carry integrated sequences from the avian retrovirus, reticuloendotheliosis virus. REV sequences in the FPV genome are not unique to Australian isolates since we have evidence for REV LTR remnants at the same site in the genomes of European vaccine and field isolates. In addition, we have characterized one European avipox virus isolate which does not have a REV LTR insertion at this location (unpublished data). For MDV, REV LTR remnants have been identified at a number of genome locations by low-stringency hybridization with LTR probes (Isfort *et al.,* 1992). So far we have not explored this possibility with FPV.

Our data show that the near-full-length REV provirus integrated into FPV S is infectious since REV was recovered when purified FPV S DNA was transfected into REV-susceptible cells. FPV M3 DNA similarly purified from cultures deliberately contaminated with REV cellular proviral DNA and free REV failed to yield REV upon transfection. This demonstrated that the REV arising from the

transfected FPV S DNA was not due to contamination since the protocol used for purification of the FPV DNAs was sufficient to remove contamination by REV cellular provirus DNA and free REV.

We were unable to demonstrate free REV in the vaccine strain FPV S; however, upon infection of susceptible chickens seroconversion occurred and REV was recovered from half of the infected chickens, suggesting that the near-full-length REV provirus in the FPV S genome was able to give rise to infectious virus *in vivo.* Low-level contamination of FPV S stocks appears unlikely since all of the chickens developed high-titer antibodies to REV and REV was recovered from 8 of 14 chickens tested after infection with FPV S. Antibody responses in those chickens from which REV was not recovered cannot be attributed to REV antigen expression without REV infection since the levels of antibody response in the virus-negative chickens was as high as those from which REV was recovered. Furthermore, our testing of FPV S as described above by RT assay, Western blotting, and immunofluorescence staining also failed to detect free REV or expression of REV antigens in the FPV S stocks. In addition, since we were unable to detect free REV in FPV S stocks, expression of the REV provirus integrated into the FPV genome appears to require *in vivo* (chicken infection) replication of FPV perhaps in cell types different from the chicken embryo fibroblast and skin cells tested so far *in vitro* and using a mechanism yet to be characterized.

Instability of the REV near-full-length provirus in the FPV genome would be anticipated since the presence of the REV LTR direct repeats would lead to intra- and intermolecular recombination (Ball, 1987). This would maintain a heterogenous genome population in those FPV isolates carrying the near-full-length REV provirus, precluding the feasibility of plaque purification to homogeneity and is reflected in the failure to obtain a full-length PCR product when flanking FPV primers were used.

Our observations add to the list of targets for retrovirus integration into DNA virus genomes. For both baculoviruses and herpesviruses integration of retrotransposons or retroviruses has been documented; however, the potential for integration and possible outcomes of integration may well be different from those of a pox virus. First, baculoviruses and herpes viruses replicate within the nucleus of infected cells, and second, gene activation following integration by the retrovirus is a possible consequence. This has already been demonstrated for REV integration into the genome of MDV (Jones *et al.,* 1996). Since poxviruses use unique promoters, gene activation by REV LTR promoter insertion appears unlikely; however, the acquisition of new or modified open reading frames by way of retrovirus integration must be considered.

A number of other possible consequences follow from retrovirus integration into poxvirus genomes. Insertional inactivation of genes could lead to modified phenotype and disease-causing capacity. We do not, as yet, have any evidence for phenotypic changes associated with the REV sequences integrated into the FPV genome. The transduction of cellular genes by retrovirus integration could facilitate the acquisition of new genes by the poxvirus. Poxviruses are now known to carry a wide range of genes, many of which have cellular homologues and many of which interact with the immune response of the host favoring virus survival and modifying the virulence and pathogenicity of the virus (Smith, 1993). Intriguingly, the FPV thymidine kinase gene is located in a region different from the region in which orthopoxvirus thymidine kinase is located. The FPV thymidine kinase gene is flanked by 15-bp direct repeats, suggesting the possibility of retrotransposition as a mechanism of independent acquisition of this gene by FPV (Binns *et al.,* 1992). Additionally vaccinia virus carries a retroviral protease-like gene which is flanked by nearly perfect 9-bp direct repeats (Slabaugh and Roseman, 1989), again suggesting the possibility of a poxvirus acquiring new genes by retroviral transposition.

FPV carrying REV appears to be circulating widely in Australia since five of five FPV isolates made in 1988 and 1989 carry near-full-length REV provirus. The vaccine strains FPV M and S were originally derived from field isolates of FPV collected in the mid-1960s, suggesting that the integration of REV into the FPV genome is not a recent occurrence. Our observation that European vaccine and field strains of FPV also carry REV LTR sequences at the same genome location suggests a wide geographical distribution and supports the conclusion that integration has not occurred recently. The integration of a near-full-length and infectious REV provirus into FPV provides additional transmission routes for the retrovirus by way of the infectious cycle of FPV, including the possibility of mechanical transmission by biting insects since poxviruses are known to be transmitted by this route. There may be a selective advantage for both viruses since immunosuppression induced by REV could prolong the duration of FPV infection and thus significantly extend the period of transmission of both viruses. For large DNA viruses, retrovirus integration with attendant possibilities of gene transduction may well be an important mechanism for virus evolution. For the retrovirus, its rate of evolution may be constrained by the rate of evolution of the poxvirus with constant seeding of the constrained retrovirus genotype through the infectious cycle of the poxvirus.

ACKNOWLEDGMENTS

REFERENCES

Aly, M. M., Smith, E. J., and Fadly, A. M. (1993). Detection of reticuloen-dotheliosis virus infection using the polymerase chain reaction. *Avian Pathol.* **22,** 543–554.

Bagust, T. J., and Dennett, D. P. (1977). Reticuloendotheliosis virus: Experimental infection of poultry and immunofluorescent identification of Australian isolates. *Aust. Vet. J.* **53,** 506–508.

Ball, L. A. (1987). High-frequency homologous recombination in vaccinia virus DNA. *J. Virol.* **61,** 1788–1795.

Binns, M. M., Boursnell, M. E. G., and Skinner, M. A. (1992). Gene translocations in poxviruses: The fowlpox virus thymidine kinase gene is flanked by 15bp direct repeats and occupies the locus which in vaccinia virus is occupied by the ribonucleotide reductase large subunit gene. *Virus Res.* **24,** 161–172.

Blissard, G. W., and Rohrmann, G. F. (1990). Baculovirus diversity and molecular biology. *Annu. Rev. Entomol.* **35,** 127–155.

Boyle, D. B., Pye, A. D., and Coupar, B. E. H. (1997). Comparison of field and vaccine strains of Australian fowpox viruses. *Arch. Virol.* **142,** 737–748.

Chen, I. S., Mak, T. W., O'Rear, J. J., and Temin, H. M. (1981). Characterisation of reticuloendotheliosis virus strain T DNA and isolation of a novel variant of reticuloendotheliosis virus strain T by molecular cloning. *J. Virol.* **40,** 800–811.

Coupar, B. E. H., Teo, T., and Boyle, D. B. (1990). Restriction endonuclease mapping of the fowlpox views genome. *Virology* **179,** 159–167.

Isfort, R. J., Jones, D., Kost, R., Witter, R., and Kung, H-J. (1992). Retrovirus insertion into herpesvirus in vitro and in vivo. *Proc. Natl. Acad. Sci. USA* **89,** 991–995.

Isfort, R. J., Qian, Z., Jones, D., Silva, R. F., Witter, R., and Kung, H. J. (1994). Integration of multiple chicken retroviruses into multiple chicken herpesviruses: Herpesviral gD as a common target of integration. *Virology* **203,** 125–133.

Jones, D., Isofort, R., Witter, R., Kost, R., and Kung, H-J. (1993). Retroviral insertions into a herpesvirus are clustered at the junction of the short repeat and short unique sequences. *Proc. Natl. Acad. Sci. USA* **90,** 3855–3859.

Jones, D., Brunovskis, P., Witter, R., and Kung, H-J. (1996). Retroviral insertional activation in a herpesvirus: Transcriptional activation of U_s genes by an integrated long terminal repeat in a Marek's disease virus clone. *J. Virol.* **70,** 2460–2467.

Lerch, R. A., and Friesen, P. D. (1992). The baculovirus-integrated retrotransposon TED encodes *gag* and *pol* proteins that assemble into viruslike particles with reverse transcriptase. *J. Virol.* **66,** 1590–1601.

Silim, A., Masy, E. A., and Roy, R. S. (1982). A simple technique for preparation of chicken-embryo-skin cell cultures. *Avian Dis.* **26,** 182–185.

Slabaugh, M. B., and Roseman, N. A. (1989). Retroviral protease-like gene in the vaccinia virus genome. *Proc. Natl. Acad. Sci. USA* **86,** 4152–5155.

Smith, G. L. (1993). Vaccinia virus glycoproteins and immune evasion. *J. Gen. Virol.* **74,** 1725–1740.

Swift, R. S., Boerkoel, C., Ridgway, A., Fujita, D. J., Dodgson, J. B., and Kung, H.-J. (1987). B-Lymphoma induction by reticuloendotheliosis virus: Characterization of a mutated chicken syncytial virus provirus involved in c-myc activation. *J. Virol.* **61,** 2084–2090.