JOURNAL OF VIROLOGY, Apr. 2001, p. 3605–3612 Vol. 75, No. 8
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.8.3605–3612.2001

Characterization of Endogenous Avian Leukosis Viruses in Chicken Embryonic Fibroblast Substrates Used in Production of Measles and Mumps Vaccines

JEFFREY A. JOHNSON AND WALID HENEINE*

HIV and Retrovirology Branch, Division of AIDS, STD, and TB Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

Received 14 November 2000/Accepted 12 January 2001

Previous findings of low levels of reverse transcriptase (RT) activity in chick cell-derived measles and mumps vaccines showed this activity to be associated with virus particles containing RNA of both subgroup E endogenous avian leukosis viruses (ALV-E) and endogenous avian viruses (EAV). These particles originate from chicken embryonic fibroblast (CEF) substrates used for propagating vaccine strains. To better characterize vaccine-associated ALV-E, we examined the endogenous ALV proviruses (*ev* loci) present in a White Leghorn CEF substrate pool by restriction fragment length polymorphism. Five *ev* loci were detected, *ev-1, ev-3, ev-6, ev-18*, and*ev-19*. Both *ev-18* and *ev-19* can express infectious ALV-E, while *ev-1, ev-3*, and *ev-6* are defective. We analyzed the full-length sequence of *ev-1* and identified an adenosine insertion within the *pol* RT-β region at position 5026, which results in a truncated RT-β and integrase. We defined the 1,692-bp deletion in the *gag-pol* region of *ev-3*, and we found that in *ev-6*, sequences from the 5′ long terminal repeat to the 5′ *pol* region were absent. Based on the sequences of the *ev* loci, RT-PCR assays were developed to examine expression of ALV-E particles (EV) in CEF supernatants. Both *ev-1*- and *ev-3*-like RNA sequences were identified, as well as two other RNA sequences with intact *pol* regions, presumably of *ev-18* and *ev-19* origin. Inoculation of susceptible quail fibroblasts with CEF culture supernatants from both 5-azacytidine-induced and noninduced CEF led to ALV infection, confirming the presence of infectious ALV-E. Our data demonstrate that both defective and nondefective *ev* loci can be present in CEF vaccine substrates and suggest that both *ev* classes may contribute to the ALV present in vaccines.

Reverse transcriptase (RT) activity, an indication of the presence of retroviruses, was recently detected in chick cell-derived live, attenuated vaccines including those produced by European and U.S. manufacturers for measles, mumps, and yellow fever (8, 32, 41). Chicken embryos and chicken embryonic fibroblasts (CEFs) from controlled breeding flocks are used in vaccine manufacture to propagate high-titer attenuated vaccine inocula. The use of chicken embryos and CEF in vaccine manufacturing requires that they be derived from closed, specific-pathogen-free source chickens that are free of known exogenous retroviral chicken pathogens, including the reticuloendotheliosis virus and the avian leukosis virus (ALV) groups (45).

Investigations of the origin of RT activity in the measles vaccine found evidence of particles containing endogenous avian virus (EAV) RNA in a vaccine manufactured in Europe (44), while evidence of both EAV and endogenous avian leukosis virus (ALV-E) was found in a vaccine made in the United States (41). While vaccine manufacturing regulations require elimination of exogenous retroviral infections from source chickens, these regulations do not address the presence of endogenous retroviruses because such particles were not previously known to be associated with chick cell-derived vaccines.

Both EAV and ALV-E are members of endogenous retrovirus families present in the chicken germ line. Little is known about the EAV family, which is distinct from but related to the ALV family. EAV elements are present in at least 50 copies per chicken genome (36). However, none of the known EAV sequences represents full-length and intact retroviral genomes, and no infectious EAV isolates have yet been identified (9).

ALV-Es are expressed from *ev* loci, which are inheritable proviral elements. Based on their envelope sequences, ALV-Es are differentiated from ALV subgroups A to D and J, which are all exogenously acquired infections (15, 35). While exogenous ALVs cause several neoplastic diseases (12, 14) and nonneoplastic diseases, such as myocarditis (20) and osteopetrosis (38), in infected chickens, ALV-Es are not known to be pathogenic to chickens (16, 17, 31). The lack of oncogenic potential with ALV-E infections may be attributed to the absence of both a viral oncogene and enhancer activity in the endogenous long terminal repeat (LTR) (18, 22, 34, 43).

The host range of ALV-E is distinct from that of exogenous ALVs. Host specificity is directed by the gp85 envelope surface protein (19, 23, 27). In chicken cells, TVB^{S1} has been identified as a receptor for ALV-E and has sequence similarities to the human tumor necrosis factor receptor-related receptors, TRAIL-R1 and TRAIL-R2 (1, 2); however, it is not known whether other receptors may be utilized. A homologous receptor, TVB^{T} or SEAR, is found in turkey cells.

More than 20 different *ev* loci have been identified in White Leghorn chickens (*ev-1* through *ev-22*). *ev* loci designations are assigned in the order discovered and are phenotypically categorized with regard to the gene products they express and their capacity to generate infectious particles. ALV-E particle (EV)

* Corresponding author. Mailing address: HIV and Retrovirology Branch, CDC, 1600 Clifton Rd., Mail Stop G-19, Atlanta, GA 30333. Phone: (404) 639-0218. Fax: (404) 639-1174. E-mail: WMH2@cdc.gov.

phenotypes conferred by *ev* loci range from structurally and enzymatically complete infectious particles (V-E$^+$) to structurally (gs$^-$[*gag*$^-$], chf$^-$[*env*$^-$]) or enzymatically (RT$^-$) defective to no detectable viral protein expression. Most *ev* loci are structurally incomplete and therefore do not encode all sequences necessary for production of infectious virus particles. Sequence mutations and deletions may arise during reverse transcription from interstrand homologous recombination between viruses of the same or similar species or from nonhomologous recombination with the host genome. In *ev-3* (gs$^+$ chf$^+$ RT$^-$), sequences surrounding the *gag-pol* junction are deleted; in *ev-5* (gs$^-$ chf$^-$ RT$^-$) and *ev-6* (gs$^-$ chf$^+$ RT$^-$), the 5′ LTR-*gag* sequences are absent (6, 23). *ev-1* (gs$^-$ chf$^-$ RT$^-$) and *ev-9* (gs$^-$ chf$^+$ RT$^-$) appear to be structurally intact yet express few or no particles (5, 6). The *ev-7* locus harbors no gross deletions and spontaneously expresses RT$^+$ particles; however, these particles are not known to be infectious (4). In contrast, several complete loci (e.g., *ev-2, ev-11, ev-12, ev-18*, and *ev-21*) yield particles which, in vitro and in vivo, are infectious to a variety of subgroup E-receptive fowl (4, 10, 13, 26, 42). In White Leghorns, *ev-1* is ubiquitous and the other loci are less prevalent and vary in number depending on the chicken line (21, 40). Any given chicken may contain several different loci. The phenotypes of *ev* loci vary with the chicken population, and therefore the phenotypes of endogenous particles present in CEF substrates may vary with the embryo pool used.

Since ALV-E particles in the U.S. measles, mumps, and rubella (MMR_{II}) vaccine originate from CEF, a better characterization of the *ev* loci present in CEF substrates is essential for determining whether these viruses are replication competent or defective. In this study, we undertook a detailed genetic and virologic analysis of the ALV-E present in a CEF substrate pool and have documented the presence of both defective and nondefective ALV-E particles.

MATERIALS AND METHODS

CEF substrate and quail fibroblasts. WG Qual. 4, a cryopreserved CEF pool of 22 White Leghorn embryos, was kindly provided by a vaccine manufacturer (Merck Research Laboratories, West Point, Pa.). One vial from WG Qual 4 was expanded by culture in growth medium specified by the vaccine manufacturer (medium 199 containing 10% tryptose broth, 8% fetal calf serum, 0.08% $NaHCO_3$, and 1% penicillin-streptomycin) in 5% CO_2. Additionally, one vial of CEF lot BPE 018 was also provided by the manufacturer as frozen, nonviable cells. The BPE 018 lot underwent only *ev* typing by *ev*-specific PCR analysis. Neither lot had been inoculated with either mumps or measles vaccine viruses. Japanese quail fibrosarcoma fibroblasts (QT35) (33) (Synpro) were grown under the same conditions as the CEF cultures. Inoculation medium has the same formulation as growth medium with the exception of containing 2% fetal calf serum.

RFLP and Southern analysis. The *ev* loci present in the WG Qual 4 CEF substrate were typed by restriction fragment length polymorphism (RFLP) analysis using previously described methods (6). To obtain genomic material, cultured CEFs were harvested by trypsinization and washed twice with phosphate-buffered saline. Aliquots of $\sim 3 \times 10^6$ cells were lysed in 600 μl of nuclei lysis buffer, and DNA was isolated with the Wizard genomic isolation kit (Promega, Madison, Wis.). Two aliquots of genomic DNA were digested overnight at 37°C, one each with *Sac*I and *Bam*HI (1 U/μg of DNA). For RFLP analysis, 30 μg of each digest was loaded into designated lanes of a 25-cm, 0.7% agarose gel and fractionated by electrophoresis at 2 V/cm for 16 h. The DNA fragments were then transferred to a Hybond N membrane (Amersham, Piscataway, N.J.) by the Southern method (39). Following transfer, the DNA was UV cross-linked to the membrane and the blot was prehybridized at 65°C for 1 h with 100 ml of 2× SET (0.05 M NaCl, 0.003 M Tris-Cl, and 0.2 mM EDTA) containing 200 mg of heparin. The digested DNA was hybridized overnight at 65°C with the 2.2-kb Multiprime (Boehringer-Mannheim) ^{32}P-labeled evpol-3′LTR probe generated from PCR amplification of CEF DNA using the evpolF8 and evLTRR2 primers (Table 1). Following hybridization, the membranes were given three 15-min washes with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate and then one wash with 0.1× SSC–0.2% sodium dedecyl sulfate at 65°C. Blots were exposed to X-Omat film (Kodak) for 7 days. Loci were identified by comparing resolved bands to a table of defined restriction patterns (14).

TABLE 1. Primers used in this study for *ev* loci and genomic RNA

Name	Sequence (5′-3′)
EAVLTRd F1	TTGTAATAGGCGTGATCGGGGT
EAVLTRd R1	GGCGTTTATTAAGTTCTAACAAGCACT
evLTRF1	GCGTAGACGAAGCTATGTACGAT
evLTRR2	TCGTCTACGCCCATATGTCCTTGCGTCAT
evLTRdF2	TGGTGGCGACTAGATAAGGAAGGAATGAC
evLTRdR2	CTGCTTCATTCAGGTGTTCGCAATCGT
ev1locF2	GGCACCAAACAATCTAGTCTGTGCT
ev3locF1	GAAATGCCTGCCCCATGCCAGTG
ev_3′UTRR1	ACTCCTAACCGCGTACAACCGAAG
ev1locR2	TCTGAACAGGATAAAGTGTGCAACAGAT
ev3locR2	CTTCTCCAGCTTCAGTGACGC
ev6locR2	TCCAAGCACTTTCCAGACACCCCAGGT
evFLF3	GTATATCACCGCGCTGTTGGACTCT
evFLR3	GTCATCCCTTGGGGCAAGACCT
ev1spF1	TTCAGCAGCTTATACGGGCAGCAC
ev3flR2	TGGAGAACGGCGGCCATGGCT
ev6envF1	TTTGAGGGATATGTCTCTGATACAA
ev6envR1	TCTCCACAGGTGTAAATATCAAAAC
ev_5′polF2	TCTGCAGGGCCTAGGGCTCCGCT
ev3delF2	CACCCATCTCTGCGCATGTTGCAT
evspF2	AGGACTGGCCCACCGATTGGCCAGT
evpolF3	TAGGAAGAGATTGTCTGCAGGGCCT
evpolF4	CCTGATGGTCCTAGACCTCAAGGAT
evpolF5	CAGAGGGAGCCCGGAGTACAATAT
evpolF8	GCGTATCCCTTGAGAGAGGCTAAAG
evpolR3	CCTTTTTCCCACTCCCCTGTCTCT
evpolnesREV	ACCTTCGGAAATAGGGGACGGGAT

***ev* typing by locus-specific PCR analysis.** Sensitive *ev* locus-specific PCR detection assays were also used in analysis of the CEF substrate to confirm the RFLP data and also to determine whether these methods could detect loci in the CEF pools which may not be detectable by RFLP. *ev* typing by PCR was also used to determine whether *ev* loci varied in the two different CEF lots. One vial of frozen CEFs ($\sim 2 \times 10^6$ cells each) from each lot were rapidly thawed in a 37°C water bath. The cells were resuspended in 500 μl of cell lysis buffer (50 mM KCl, 10 mM Tris HCl [pH 8.3], 0.01% gelatin, 0.45% NP-40, 0.45% Tween 20, 100 μg of proteinase K/ml) and incubated at 56°C for 1 h. The lysate was then boiled for 10 min to inactivate the proteinase K. Endogenous *ev* loci within the CEF genomic DNA were detected based on the methods of Benkel (7), using 10 μl (~0.25 μg) of the DNA lysate. Briefly, PCR primers (Fig. 1; Table 1) directed against the *ev* LTRs and the sequences flanking known integration sites for each locus were used. Amplification of the targeted sites produced fragments of defined lengths which indicated the presence (an LTR to locus-flanking sequence fragment) or absence (a chicken-only sequence fragment) of the locus. We tested for eight loci for which assays were available and which are known to yield particles and/or RT activity and to be prevalent within White Leghorn chickens. These assays included tests for the *ev-1, ev-2, ev-3, ev-6, ev-7, ev-9, ev-12*, and *ev-21* loci (7). The annealing temperatures for the final set of cycles in the modified touchdown PCRs were 48°C for *ev-1*; 54°C for *ev-2, ev-3, ev-7*, and *ev-12*; 60°C for *ev-6*; and 53°C for *ev-9* and *ev-21*.

Long-template PCR amplification of *ev-1, ev-3*, and *ev-6*. To analyze *ev-1* and *ev-3*, specific amplification of each locus was performed by long-template PCRs (Boehringer-Mannheim). Primers ev1locF2 (modified from reference 7) and ev3locF1 (Table 1), complementary to chicken sequences flanking the 5′ ends of *ev-1* and *ev-3*, respectively, were each used in conjunction with a common 3′ untranslated region primer (ev_3′UTRR1) (Fig. 1). This generated sequences from the 5′ flanks to the 3′ untranslated regions of the *ev-1* and *ev-3* loci. To obtain the remaining 3′ ends of both loci, primers ev1locR2 and ev3locR2 were used in conjunction with evpolF8 to amplify the sequence from *pol* to the

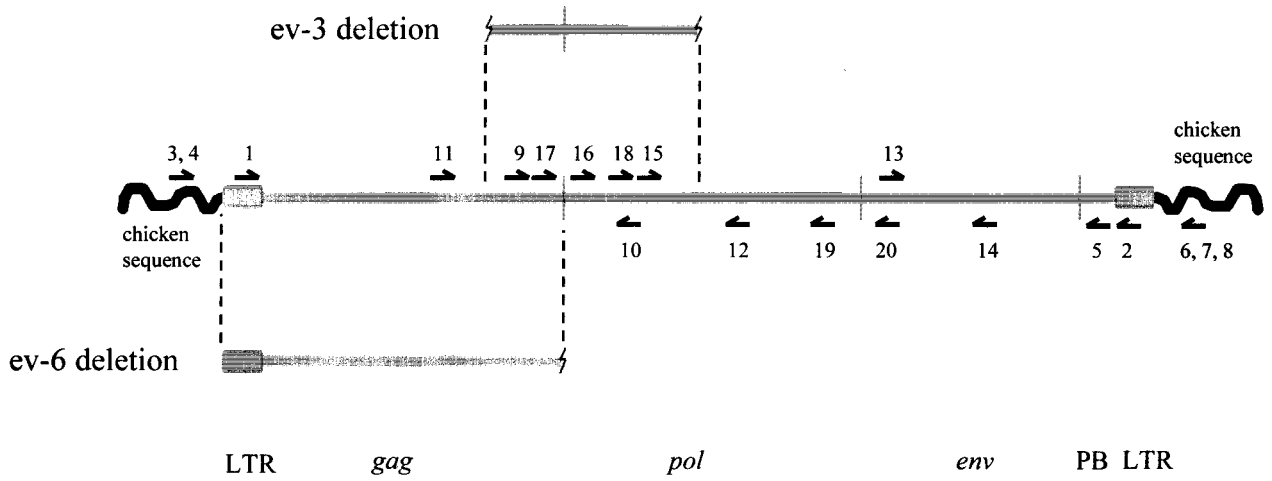

FIG. 1. Schematic of *ev* loci and regions of primer hybridization used in the detection and amplification of *ev* sequences. 1, evLTRF1; 2, evLTRR2; 3, ev1locF2; 4, ev3locF1; 5, ev_3′UTRR1; 6, ev11ocR2; 7, ev3locR2; 8, ev6locR2; 9, evFLF3; 10, evFLR3; 11, ev1spF1; 12, ev3flR2; 13, ev6envF1; 14, ev6envR1; 15, ev3delF2; 16, ev_5′polF2; 17, evspF2; 18, evpolF4; 19, evpolR3; 20, evpolnesREV. The regions deleted in the genomes of *ev-3* and *ev-6* are illustrated above and below the full-length locus, respectively.

3′-flanking chicken sequences of *ev-1* and *ev-3*, respectively. Long-template PCRs were performed using an annealing temperature of 59°C and extensions for 8 min at 70°C for 35 cycles. Amplification of *ev-6* involved using several PCRs with different forward primers, complementary to the 3′ *gag* and 5′ *pol* regions, with the ev6locR2 reverse primer, which annealed to the 3′ end of the *ev-6* locus. The forward primers evspF2, ev_5′polF2, and evpolF4 were used in an attempt to amplify the entire locus and delineate the 5′ end of *ev-6*.

Cloning and sequence analysis. PCR-amplified sequences were ligated into the TA cloning vector (Invitrogen, Carlsbad, Calif.) overnight at 16°C. Top 10 F' One Shot (Invitrogen) chemically competent *Escherichia coli* cells (provided with the kit) were heat shock transformed with the ligated vectors and then spread on Luria-Bertani plates containing isopropyl-β-D-thiogalactopyranoside (IPTG), β-galactosidase, and 50 μg of kanamycin per ml. White colonies were selected from the culture plates and tested by colony PCR assays for the sequence of interest. All sequences underwent double-strand analysis by the chain termination method using the Big Dye Terminator (Perkin-Elmer, Foster City, Calif.) reagents. Sequencing reactions were performed for 25 cycles of melting at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 1 min, and the products were electrophoresed using a 373 Stretch automated sequencer (Applied Biosystems/Perkin-Elmer).

Extraction of particle-associated RNA. CEF culture supernatants were clarified by centrifugation at 500 × *g* for 10 min. Virus particles were harvested from 100 ml of clarified culture supernatant by ultracentrifugation at 100,000 × *g* for 1 h. After ultracentrifugation, particle pellets were resuspended in Dulbecco's phosphate-buffered saline and pooled into a total volume of 200 μl. Free RNA and DNA were digested with DNase (5 U) and RNase (2 U) for 1 h and 15 min, respectively, at 37°C. The RNase and DNase were inactivated by the viral RNA extraction-solubilization buffer from the QIAamp Viral RNA kit (Qiagen, Inc.), which was used to extract particle-associated RNA.

ALV-E RNA typing by EV-specific RT-PCR assays. To type particle-associated RNA from CEF supernatants, we developed specific RT-PCR assays for either *ev-1*/full-length *pol, ev-3*, or *ev-6*. The *ev-3*-specific assay uses primers ev1spF1 and ev3flR2, which flank the deletion in *ev-3* and yield a 761-bp fragment that is diagnostic of *ev-3*. The evFLF3 and evFLR3 primer set (Table 1) amplified exclusively *ev-1* and any other potentially full-length *pol* genomic RNA sequences. The primers that identify the *ev-6 env* region are ev6envF1, which is complementary to a sequence that includes a trinucleotide insert characteristic of *ev-6*, and ev6envR1, which flanks two other base changes specific for the *ev-6* envelope (see Results). All RT-PCR reactions were performed on 250 ng of the particle-associated RNA and included an RT-negative control reaction using *ev-3* specific primers. The reverse transcription reactions were carried out for 60 min at 37°C using 50 U of murine leukemia virus RT (Perkin-Elmer). PCRs were performed for 35 cycles (melting at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min). The amplified DNA was Southern blot hybridized to the ^{32}P-end-labeled oligonucleotide probes evgag-pol.1P, evmidpol.1P, and ev6spenv.1P for the *ev-1, ev-3*, and *ev-6* products, respectively. The sequences of the primers and probes are listed in Tables 1 and 2. Relative levels of the different EV RNA types in the CEF culture supernatant were determined by end-point dilution *ev*-specific RT-PCR amplification.

Clonal analysis of full-length *pol* sequences from EV RNA in the CEF supernatant. To determine the number of different RNA species in CEF that have full-length *pol* sequences, EV RNA was first amplified by long-template RT-PCR using the primer pair evspF2 and evpolnesREV, which yielded a 3,139-bp fragment. A 1,909-bp fragment, which included the signature mutation of *ev-1* as well as other sequences useful for comparison, was subsequently amplified with the nested primers ev3delF2 and evpolR3, and the product was cloned and sequenced as described above.

Inoculation of quail cells with supernatants from azacytidine-induced and non-induced CEF cultures. Third-passage WG Qual. 4 CEFs were seeded in two sets of duplicate T-162 culture flasks and grown to approximately 50% confluence. The cells in one set of flasks were allowed to continue growing without modification of the growth medium. Since *ev* loci are generally weakly expressed due to the repression of transcription via methylation of flanking CpG islands, a second set of CEF cultures was incubated for 24 h with 3 μM 5-azacytidine (a demethylating agent) to induce *ev* RNA expression. The cultures were then washed and refreshed with normal growth medium and incubated for 72 h. Culture supernatants (50 ml/set) were harvested and ultracentrifuged for 1 h at 100,000 × *g*. Pellets from each set were resuspended in 4 ml of inoculation medium and inoculated onto QT35 Japanese quail fibrosarcoma cultures grown to 10% confluence in T-25 flasks. A flask of quail cells was also inoculated with a mock control of inoculation medium only. Cells were incubated with the inocula for 48 h and were then washed and refreshed with normal growth medium. Cultures were harvested by trypsinization, and 20% was reseeded in T-75 flasks on days 5, 11, 19, 25, and 29. The remaining 80% of each cell culture was collected for DNA analysis. Culture supernatants were collected at these time points and at the intervening weekly culture refreshments.

TABLE 2. *ev* probes used in this study

Name	Sequence (5′-3′)
EAVLTRd.1P	GTACACGAAGGGTTAAAAGATATAT
ev gag-pol.1P	TAACGCAATTAGTGGAAAAAGAAT
ev midpol.1P	TTTGTTGCGAAAATGTTACTCAAG
ev p19.2P	TTAGTTATGGCAGTAGTCAATAGA
evLTRd.2P	TAGACGAAGCTATGTACGATTATA
evLTRA.1P	GATATTAAAGTCAATTTCTACTAAG
ev6spenv.1P	CTAATATTACTCAGATTTCTGGTGT

Testing for ALV-E infection in inoculated quail fibroblasts. Cells were screened for ALV infection by ALV LTR PCR amplification of extracted DNA using the evLTRdF2 and evLTRdR2 primer set (Table 1). PCR products were detected by Southern blot hybridization to the ^{32}P evLTRd.2P probe (Table 2). Additionally, all samples were screened for two chicken DNA sequences to exclude contamination by residual CEF DNA. Primers evLTRF1 and ev1locR2, based on the *ev-1* LTR and flanking chicken sequence, were used to screen for the ubiquitous *ev-1* locus-specific sequence. Additionally, the EAVLTRd F1 and EAVLTRd R1 primers (Table 1) were used to screen for EAV sequences which exist in high copy numbers in chicken DNA. The PCR products were probed with evLTRA.1P and EAVLTR.1P for the *ev* and EAV sequences, respectively.

Quail culture supernatants were screened for productive ALV expression by detecting RT activity by the PCR-based Amp-RT assay as described previously (25). Briefly, 10 μl of clarified supernatants was diluted 1:2 in RT buffer containing a 350-bp encephalomyocarditis virus (EMCV) RNA template and 200 ng of a complementary reverse primer. The mixture was incubated at 37°C for 2 h and then inactivated by heating at 95°C. The reaction-generated EMCV cDNA was amplified by PCR using 2.5 U of *Taq* polymerase and a forward primer for the EMCV sequence. Amp-RT products were detected by Southern blot hybridization as previously described (25). Tests were performed on supernatants from both 5-azacytidine-induced and control quail fibroblasts. The quail supernatants tested represented samples collected at 11, 19, 25, 27, and 29 days after CEF inoculation.

Particle-associated RNA was examined for the presence of ALV-E *env* and *pol* sequences. Characterization of particle-associated ALV RNA was performed by sequence analysis of *pol* following RT-PCR amplification of the complete 3-kbp *pol* region. The evpolF3 and evpolnesREV primers were used in a primary RT-PCR, and the sequence of interest was amplified by nested PCR using the evpolF5 and evpolR3 primers (Table 1). The nested PCR product was cloned and sequenced as described above.

Probe labeling. A 200-ng portion of each oligonucleotide probe was end labeled at 37°C for 30 min with 2 U of T4 polynucleotide kinase (New England Biolabs) and 50 μCi of [γ-^{32}P]ATP in a total volume of 50 μl. Probes were purified on Sephadex G-50 spin columns (Pharmacia Biotech) to remove unbound [γ-^{32}P]ATP. Hybridizations were performed using 67 ng of the labeled oligonucleotide probe. For randomly labeled probes, 3 ng of the 2.2-kb *pol*-LTR fragment, generated from CEF genomic DNA, were Multiprime labeled for 3 h at room temperature using 100 μCi of [α-^{32}P]dCTP as specified for the Multiprime random labeling kit.

Nucleotide sequence accession number. The complete *ev-1, ev-3*, and *ev-6* sequences were deposited in GenBank under accession numbers AY013303, AY013304, and AY013305, respectively.

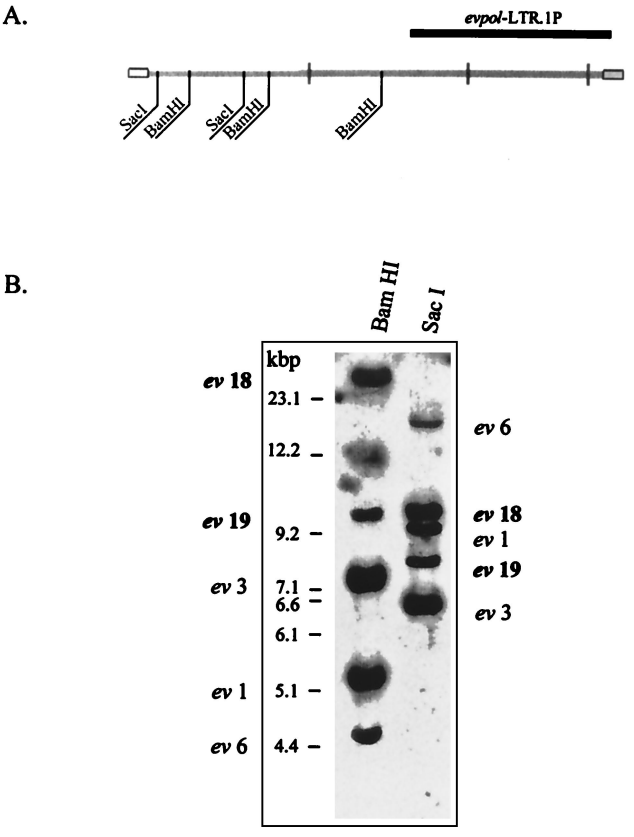

FIG. 2. RFLP analysis of *Bam*HI-*Sac*I digested DNA from vaccine substrate CEFs. (A) Restriction map schematic of *Bam*HI and *Sac*I restriction sites found in full-length *ev* loci. The location and size of the pan ev*pol*-3′LTR random-labeled probe is shown. (B) Diagnostic *ev-1, ev-3, ev-6, ev-18*, and *ev-19* bands detected in the *Bam*HI and *Sac*I digests.

RESULTS

RFLP analysis of CEF genomic DNA. Southern blot hybridization of *Bam*HI-digested CEF DNA from the WG Qual. 4 vaccine substrate lot with the evpol-3′LTR probe produced bands of 25, 9.8, 7.3, 5.1, and 4.3 kbp, which corresponded to *ev-18, ev-19, ev-3, ev-1*, and *ev-6*, respectively (Fig. 2). The *Sac*I digest yielded bands of 21, 10.5, 9.3, 7.6, and 6.3 kbp, which corresponded to *ev-6, ev-18, ev-1, ev-19*, and *ev-3*, respectively (15). Thus, the loci detected with the *Bam*HI digest were corroborated by the restriction pattern of the *Sac*I digest.

Identification of loci by *ev*-specific PCR analysis. PCR analysis of both BPE 018 and WG Qual. 4 CEF lots using locus-specific primers for *ev-1, ev-2, ev-3, ev-6, ev-7, ev-9, ev-12*, and *ev-21* showed that both lots were positive for *ev-1, ev-3*, and *ev-6* loci and negative for *ev-2, ev-7, ev-9, ev-12*, and *ev-21* loci. Therefore, these sensitive assays did not reveal additional loci and have confirmed the presence of three loci detected by RFLP (data not shown).

Sequence analysis of *ev-1, ev-3*, and *ev-6*. *ev-1* and *ev-3* sequences were successfully amplified from the WG Qual. 4 CEF lot by long-template PCR, and both genomes were sequenced. The analysis of the entire 7,525-bp *ev-1* locus revealed an adenosine insertion 2,427 bp into the *pol* open reading frame (ORF) at position 5026. This additional residue produces a frameshift in the reading frame, which causes a premature termination codon to appear 41 bp downstream of the mutation and 298 bp upstream of the *pol* polyprotein wild-type stop codon. Neither *ev-3* nor *ev-6* has the additional adenosine present in *pol*. No other deletions or insertions were identified in the *ev-1* sequence relative to the *ev-2* sequences available. Table 3 shows the sequence homologies between *ev-1* and available sequences from both exogenous ALV (ALV-A) (GenBank accession numbers M37980 and AF247392), avian myeloblastosis virus (AMV) (GenBank accession number

TABLE 3. Comparison of *ev-1* proviral sequences with *ev-2, ev-3, ev-6*, ALV-A, and AMV

Virus	% Sequence similarity to *ev-1* genomic region:			
	Complete genome	*pol*	*env*	LTR
RAV-0 (*ev-2*)	—[a]	—	99.1	99.6
ev-3	—	—	99.8	99.6
ev-6	—	—	99.1	99.2
ALV-A	90.5	99.2	89.1	55.4
AMV	88.4	97.8	87.0	54.0

[a] Sequences were not available for comparison, or gross deletions made the comparison invalid.

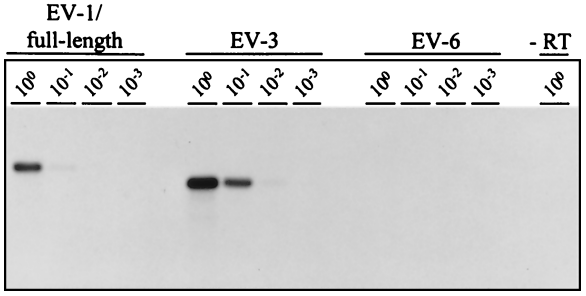

FIG. 3. Diagnostic RT-PCR of EV RNA extracted from uninoculated CEF supernatant. The undiluted RNA (10^0) is equivalent to 4 ml of culture supernatant. RT-PCRs were performed on 10-fold dilutions using the *ev-1, ev-3*, and *ev-6* diagnostic primers. −RT, reverse transcriptase-minus control.

L10924), and an endogenous ALV (*ev-2*) (Genbank accession numbers M73497, J02016, and M12172). Overall, the *ev-1* sequence was found to be highly related to both exogenous ALV (90.5%) and AMV (88.4%). Analysis by gene region also demonstrated high similarities, ranging between 87 and 99.6%, for all viral sequences except for the exogenous LTR region, which had only ~55% similarity to the *ev-1* LTR.

Analysis of the *ev-3* genome in the *gag-pol* region defined a large deletion flanked by the ev1spF1 and ev3flR2 primers. Compared to *ev-1*, the deletion spanned 1,692 bp, beginning at position 2312 and ending at position 4004, 1,405 bp into the *pol* ORF. Although protease and RT-α are disrupted in *ev-3*, the *pol* reading frame is maintained. A 17-bp fragment (GTTTA TATAAACATAAA), not homologous to ALV-E, is present at the site of the deletion and may be the remnant of a recombinatorial event that created the large deletion. Additionally, the *ev-3 env* transcript has an in-frame 6-bp deletion, 54 bp upstream of the beginning of the coding sequence for the receptor binding domain (SU, gp85).

Sequence analysis of the 3′ *pol*-LTR regions of *ev-6* revealed a 3-nucleotide (CAA) insertion 158 bp into the *ev-6 env* ORF at position 5593 relative to the complete *ev-1* sequence, as well as two A-to-G base transitions at positions 5482 and 5584. The insertion is not present in either *ev-1* or *ev-3*. We attempted to define the 5′ end of *ev-6* by using several PCR amplifications which had different forward primers paired with the same ev6locR2 primer. The results of these PCR tests indicate that the 5′ end of the *ev-6* genome begins around 200 to 400 bp 3′ of the *pol* ORF start (data not shown).

EV RNA in CEF culture supernatants. Analysis of particle-associated RNA in the CEF supernatants by the EV-specific assays revealed the presence of more than one EV RNA type. A 767-bp *ev-1*/full-length *pol* fragment and a 745-bp *ev-3*-specific sequence were both observed. In contrast to *ev-1*/full-length *pol* and *ev-3, ev-6* RNA was infrequently detected, and then only in undiluted RNA preparations, suggesting that little or no *ev-6* RNA was being packaged (Fig. 3). Titer determinations of supernatant RNA indicated that RNA levels were approximately 10-fold higher for *ev-3* than for *ev-1* (Fig. 3). All control RT-PCRs which excluded RT were negative, confirming that the amplified sequences were all of RNA origin and were not due to contamination with residual DNA.

Analysis of particle-associated RNA clones reveals three full-length *pol* species. To determine whether one or more full-length *pol* RNA species had been amplified from the supernatant of substrate WG Qual. 4, we cloned and analyzed the sequences of 20 individual clones for the presence of nucleotide changes, including the adenosine insertion that is indicative of *ev-1*. Three clones (15%) had intact sequences that did not have the adenosine insertion. Two of these clones had three nucleotide substitutions (C to T, G to A, and C to T) at positions 3796, 3900, and 4204 relative to the complete *ev-1* locus, respectively. The third sequence did not have the three nucleotide substitutions and, with the exception of the absent additional A at position 5026, showed 100% homolgy to the *ev-1* sequence. The remaining 17 (85%) clones all had the adenosine insertion and 100% sequence identity to *ev-1*. Thus, the data indicate the presence of *ev-1*-like sequences, as well as two other full-length *pol* EV RNA species in the supernatants. Given the loci detected by RFLP, the data strongly suggest that the other two full-length-*pol* EV species are of *ev-18* and *ev-19* origin.

Evidence of infectious ALV-E in CEF culture supernatants. Quail cells inoculated with supernatants from either the 5-azacytidine-induced or the uninduced CEF cultures were analyzed by PCR for the presence of ALV-E DNA. A weakly positive ALV-E PCR signal was detected in quail cells 5 days after inoculation with uninduced CEF supernatant (Fig. 4). A strong ALV-E PCR signal was observed 11 days postinoculation and persisted in the remainder of the samples tested up to day 29. Similarly, for the quail culture inoculated with the supernatant from the 5-azacytidine-treated CEF, strong PCR signals for ALV were seen by day 5 postinoculation and in all other samples tested throughout the 29-day assay period. No ALV-E sequences were detected in the mock infection control cultures. Tests for the presence of both EAV LTR and the *ev-1*/flank chicken sequences in the inoculated quail cultures were negative at all time points. This demonstrated that the observed ALV-E sequences were not due to the presence of residual chicken DNA but, rather, were due to new ALV infections (Fig. 4). The stronger ALV PCR signals in the quail culture inoculated with the 5-azacytidine-induced supernatant reflected higher levels of infectious ALV-E.

RT activity was evident 25 days postinoculation in cultures inoculated with induced supernatant and persisted with an increasing titer that indicated a productive expression of retroviral particles (Fig. 4). The quail cultures inoculated with the uninduced CEF supernatant produced weak Amp-RT signals beginning day 27 postinoculation (data not shown). The low RT activity in this culture was consistent with the weaker proviral signals and suggested a lower level of infection and expression.

Evidence of EV RNA in the supernatants of inoculated quail cultures. RT-PCR analyses of particle-associated RNA isolated from quail culture supernatants 29 days following both the uninduced and induced CEF supernatant inoculations were positive for ALV-E envelope RNA sequences (data not shown). To clone and type the EVs expressed from inoculated quail cultures, we performed nested PCR amplification of a full-length *pol* RT-PCR product on EV RNA expressed from cultures inoculated with induced CEF supernatant. From a full-length *ev pol* RT-PCR on day 29 supernatant, a 1,771-bp

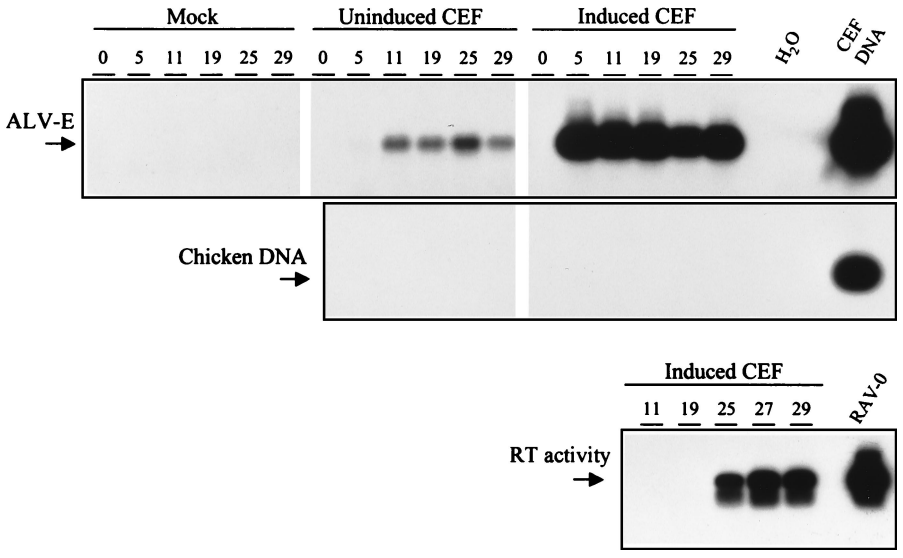

FIG. 4. ALV-E infection of CEF supernatant-inoculated quail cells. (Top) Proviral ALV-E PCR of quail DNA following inoculation with mock infection control and uninduced and 5-azacytidine-induced CEF supernatants. (Middle) PCR of quail DNA for the presence of residual chicken DNA using EAV LTR primers. (Bottom) RT activity detection by the PCR-based Amp-RT assay following inoculation with supernatants from 5-azacytidine-induced CEF. The number of days following inoculation is indicated above each lane; day 0 indicates uninoculated cells. CEF DNA, positive PCR control using 200 ng of CEF DNA; RAV-0, RT-positive ALV-E isolate used as positive control; H_2O, water negative control.

fragment was amplified by nested PCR with the evpolF5 and evpolR3 primers (Fig. 5). The fragment was cloned, and sequence analysis of 14 clones revealed two different sequences, which we believe to be *ev-18* and *ev-19*, since neither had the superfluous adenosine indicative of *ev-1*. Of 14 clones, 8 possessed the three T-A-T nucleotide substitutions previously identified in the EV RNA species found in the CEF supernatant. Six clones did not possess the three-base substitutions and had sequences that were similar to *ev-1* but lacked the adenosine insertion characteristic of *ev-1*.

DISCUSSION

This study was designed to characterize the endogenous ALV in CEF substrates used in the production of measles and mumps vaccines. Our RFLP data document the presence of *ev-1*, *ev-3*, and *ev-6*, as well as *ev-18* and *ev-19* loci, demonstrating that both defective and nondefective loci can be present in vaccine substrates. We also show that these loci are not latent or inactive, since particles that were associated with both defective *ev-1* and *ev-3* RNA and also with apparently intact sequences, probably of *ev-18* and *ev-19* origin, were expressed in CEF supernatants. The ability of the CEF supernatant to infect quail cells demonstrates the presence of infectious ALV-E, and the sequence analyses suggest that these viruses are *ev-18* and *ev-19*.

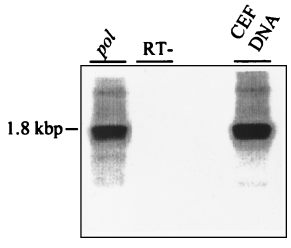

FIG. 5. ALV-E particle-associated RNA sequences expressed from quail cells inoculated with culture supernatant from azacytidine-stimulated CEF. Particle-associated RNA from day 29 supernatant was analyzed for the presence of full-length *ev pol* sequences. The 1.8-kbp fragment shown was generated from a nested PCR. RT−, RT-minus assay negative control; CEF DNA, CEF DNA PCR positive control.

Sequence analyses of the *ev-1, ev-3*, and *ev-6* loci has defined mutations or deletions which preclude them from forming replication-competent particles. The *ev-1* locus was found to have a +1 frameshift mutation in the *pol* RT-β region, which results in a truncated RT-β subunit and integrase. As a result, the truncated β subunit may be unable to dimerize with the α chain to form a functional heterodimer with an RT active site; this could explain the lack of RT activity associated with EV-1 particles noted in previous studies (11, 24). Since this mutation also truncates the integrase, which is also necessary for infectivity, the noninfectious nature of *ev-1* can be attributed to both defective RT and integrase proteins.

Our data also define the gross deletions previously described in both *ev-3* and *ev-6* (6, 24). With a large (1,692-bp) deletion in *gag-pol, ev-3* is not able to express protease and RT polymerase-α. Similarly, our results show that *ev-6* has a 5′ deletion that extends into *pol*, thereby preventing the expression of both *gag* and RT. Interestingly, the *ev-6 env* has a trinucleotide insertion identical to that seen in *ev-2*, suggesting a possible recombination event or a common lineage.

The identification of signature mutations or deletions in the sequences of *ev-1, ev-3*, and *ev-6* allowed us to develop *ev*-specific PCR assays which were used to investigate the types of ALV-E expressed in the CEF supernatant. Although virion expression is generally low with endogenous ALV, we were able to show that a mixture of different RNA species are associated with particles, including both *ev-1* and *ev-3* and two

other RNA species with intact *pol* sequences, strongly suggestive of *ev-18* and *ev-19*. Since the sequences of *ev-18* and *ev-19* are not known, it will be difficult to determine which of the two loci is responsible for each RNA sequence. Although cellular expression of *ev-6* RNA is constitutive, the predominant absence of particle-associated *ev-6* may be explained by the deleted Ψ signal sequence which is required for efficient packaging of genomic RNA (3, 30).

The infectivity data from quail cell inoculations clearly demonstrated the presence of infectious ALV-E in the CEF supernatant. The slow expansion of ALV-infected quail cells supports the finding that a very low level of infectious EV is present in the CEF supernatant. As expected with 5-azacytidine induction, the kinetics and level of the ALV-E infection in quail cells were enhanced, a consequence of the higher level of ALV-E present in the inoculum. The finding of sequences suggestive of *ev-18* and *ev-19* in the ALV-infected quail cells confirms the infectious capacity of these viruses and is consistent with the molecular evidence that EV-18 and EV-19 RNA was present in the CEF supernatant.

Our findings of replication-competent ALV-E in the vaccine substrate raises the possibility that such viruses could be present in vaccines. While chick cell-derived MMR vaccines were found to have particle-associated ALV-E RNA sequences, the specific *ev* source of these particles has not been studied (41). The presence of infectious ALV-E in different CEF preparations may vary with the prevalence of nondefective *ev* loci in the source chickens. Hence, pooling the large number of embryos required to prepare each CEF lot can increase the likelihood that a CEF preparation will contain *ev* loci that express infectious ALV-E.

The possibility that recipients of the MMR vaccine may be exposed to nondefective ALV-E highlights the importance of assessing potential risks of transmitting ALV-E to vaccine recipients. In a recent study of 206 recipients of the U.S.-made MMR_{II} vaccine, we were unable to find evidence of ALV-E infection by serologic and molecular testing, which suggests that the risks of ALV-E transmission may be low (28). While reassuring, these data are limited by the absence of information regarding whether these vaccine recipients were exposed to infectious ALV-E and whether humans are indeed susceptible to ALV-E infections. Two recent studies showed that inoculation of different human cell types with CEF supernatants did not result in ALV infection or in detectable RT activity (29, 37). However, it is not known whether the CEF supernatants used contained replication-competent ALV-E. Therefore, our study underscores the importance of screening chick cell-derived MMR vaccines for evidence of potentially infectious ALV-E and determining the susceptibility of human cells to ALV-E infection.

The detection of nondefective ALV-E in the CEF substrate raises questions whether a change to a substrate that does not express infectious ALV-E is desirable. Selecting either line 0 chickens, which do not possess ALV-E sequences, or CEF from chicken flocks screened to eliminate infectious *ev* loci would provide vaccine substrates that do not express infectious ALV-E. Although such substrates will probably express RT-positive EAV, these are believed to be defective particles. Likewise, quail cells, which are not known to express infectious retroviruses, may provide an alternate avian substrate for vaccine manufacture. However, utilizing an RT-negative substrate may require switching to cells of nonavian species, such as immortalized or diploid mammalian cells. Since the cell substrate is critical to the attenuation of live vaccine viruses, a switch in the cell substrate may have unforeseen effects on the safety and efficacy of the vaccine. Therefore, consideration of alternate substrates must be approached with caution.

ACKNOWLEDGMENTS

We are grateful to Alan Shaw and colleagues at Merck Research Laboratories for providing CEF substrates and information on their culturing, to Althaf Hussain for performing the Amp-RT analysis, and to Thomas M. Folks and Alison Mawle for critical review of the manuscript.

REFERENCES

1. **Adkins, H. B., J. Brojatsch, J. Naughton, M. M. Rolls, J. M. Pesola, and J. A. T. Young.** 1997. Identification of a cellular receptor for subgroup E avian leukosis virus. Proc. Natl. Acad. Sci. USA **94:**11617–11622.
2. **Adkins, H. B., J. Brojatsch, and J. A. T. Young.** 2000. Identification and characterization of a shared TNFR-related receptor for subgroup B, D, and E avian leukosis viruses reveal cysteine residues required specifically for subgroup E viral entry. J. Virol. **74:**3572–3578.
3. **Aronoff, R., and M. Linial.** 1991. Specificity of retroviral RNA packaging. J. Virol. **65:**71–180.
4. **Astrin, S. M.** 1978. Endogenous viral genes of the White Leghorn chicken: common site of residence and sites associated with specific phenotypes of viral gene expression. Proc. Natl. Acad. Sci. USA **75:**5941–5945.
5. **Astrin, S. M., H. L. Robinson, L. B. Crittenden, E. G. Buss, J. Wyban, and W. S. Hayward.** 1980. Ten genetic loci in the chicken that contain structural genes for endogenous avian leukosis viruses. Cold Spring Harbor Symp. Quant. Biol. **44:**1105–1109.
6. **Baker, B., H. L. Robinson, H. E. Varmus, and J. M. Bishop.** 1981. Analysis of endogenous avian retrovirus DNA and RNA: viral and cellular determinants of retrovirus gene expression. Virology **114:**8–22.
7. **Benkel, B. F.** 1998. Locus-specific diagnostic tests for endogenous avian leucosis-type viral loci in chickens. Poult. Sci. **77:**1027–1035.
8. **Böni, J., J. Stalder, F. Reigel, and J. Schüpbach.** 1996. Detection of reverse transcriptase activity in live attenuated vaccines. Clin. Diagn. Virol. **5:**43–55.
9. **Boyce-Jacino, M. T., K. O'Donoghue, and A. J. Faras.** 1992. Multiple complex families of endogenous retroviruses are highly conserved in the genus *Gallus*. J. Virol. **66:**4919–4929.
10. **Chen, Y. C., and P. K. Vogt.** 1977. Endogenous leukosis viruses in the family Phasianidae. Virology **76:**740–750.
11. **Conklin, K. F., J. M. Coffin, H. L. Robinson, M. Groudine, and R. Eisenman.** 1982. Role of methylation in the induced and spontaneous expression of the avian endogenous virus *ev-1*: DNA structure and gene products. Mol. Cell. Biol. **2:**638–652.
12. **Cooper, M. D., L. N. Payne, P. B. Dent, B. R. Burmester, and R. A. Good.** 1968. Pathogenesis of avian lymphoid leukosis. I. Histogenesis. J. Natl. Cancer Inst. **41:**373–378.
13. **Crittenden, L. B., W. Okazaki, and E. J. Smith.** 1983. Incidence of avian leukosis virus infection in broiler stocks and its effect on early growth. Poult. Sci. **62:**2383–2386.
14. **Crittenden, L. B., W. S. Hayward, H. Hanafusa, and A. M. Fadly.** 1980. Induction of neoplasms by subgroup E recombinants of exogenous and endogenous avian retroviruses (Rous-associated virus type 60). J. Virol. **33:**915–919.
15. **Crittenden, L. B.** 1991. Retroviral elements in the genome of the chicken: implications for poultry genetics and breeding. Crit. Rev. Poult. Biol. **3:**73–109.
16. **Crittenden, L. B., R. L. Witter, and A. M. Fadly.** 1979. Low incidence of lymphoid tumors in chickens continously producing endogenous virus. Avian Dis. **23:**646–653.
17. **Crittenden, L. B., R. L. Witter, W. Okazaki, and P. E. Neiman.** 1979. Lymphoid neoplasms in chicken flocks free of infection with exogenous avian tumor viruses. JNCI **63:**191–200.
18. **Cullen, B. R., K. Raymond, and G. Ju.** 1985. Functional analysis of the transcription control region located within the avian retroviral long terminal repeat. Mol. Cell. Biol. **5:**438–447.
19. **Dorner, A. J., and J. M. Coffin.** 1986. Determinants for receptor interaction and cell killing on the avian retrovirus glycoprotein gp85. Cell **45:**365–374.
20. **Gilka, F., and J. L. Spencer.** 1990. Chronic myocarditis and circulatory syndrome in a White Leghorn strain induced by an avian leucosis virus: light and electron microscope study. Avian Dis. **34:**174–184.
21. **Gudkov, A. V., I. B. Obukh, S. M. Serov, and B. S. Naroditsky.** 1981. Variety of endogenous proviruses in the genomes of chickens of different breeds. J. Gen. Virol. **57:**85–94.

22. **Habel, D. E., K. L. Dohrer, and K. F. Conklin.** 1993. Functional and defective components of avian endogenous virus long terminal repeat enhancer sequences. J. Virol. **67:**1545–1554.
23. **Hadwiger, A., and J. V. Bosch.** 1985. Characterization of the endogenous retroviral envelope glycoproteins found in the sera of ev3 and ev6 chickens. J. Gen. Virol. **66:**2051–2056.
24. **Hayward, W. S., S. B. Braverman, and S. M. Astrin.** 1980. Transcriptional products and DNA structure of endogenous avian proviruses. Cold Spring Harbor Symp. Quant. Biol. **44:**1111–1121.
25. **Heneine, W., S. Yamamoto, W. M. Switzer, T. J. Spira, and T. M. Folks.** 1995. Detection of reverse transcriptase by a highly sensitive assay in sera from persons infected with human immunodeficiency virus type 1. J. Infect. Dis. **171:**1210–1216.
26. **Humphries, E. H., M. L. Danhof, and I. Hlozanek.** 1984. Characterization of endogenous viral loci in five lines of White Leghorn chickens. Virology **135:**125–138.
27. **Hunter, E., E. Hill, M. Hardwick, A. Bhown, D. E. Schwartz, and R. Tizard.** 1983. Complete sequence of the Rous sarcoma virus *env* gene: identification of structural and functional regions of its product. J. Virol. **46:**920–936.
28. **Hussain, A. I., V. Shanmugam, W. M. Switzer, S. X. Tsang, A. Fadly, D. Thea, R. Helfand, W. J. Bellini, T. M. Folks, and W. Heneine.** Lack of evidence of transmission of endogenous avian leukosis virus and endogenous avian retrovirus to recipients of measles, mumps and rubella vaccine. Emerg. Infect. Dis., in press.
29. **Khan, A. S., T. Maudru, A. Thompson, J. Muller, J. F. Sears, and K. W. C. Peden.** 1998. The reverse transcriptase activity in cell-free medium of chick embryo fibroblast cultures is not associated with a replication-competent retrovirus. J. Clin. Virol. **11:**7–18.
30. **Linial, M. L., and A. D. Miller.** 1990. Retroviral RNA packaging: sequence requirements and implications. Curr. Top. Microbiol. Immunol. **157:**125–152.
31. **Linial, M., and P. E. Neiman.** 1976. Infection of chick cells by subgroup E viruses. Virology **73:**508–520.
32. **Maudru, T., Cooperating Units, and K. W. C. Peden.** 1998. Analysis of a coded panel of licensed vaccines by polymerase chain reaction-based reverse transcriptase assays: a collaborative study. J. Clin. Virol. **11:**19–28.
33. **Moscovici, C., M. G. Moscovici, H. Jimenez, M. M. Lai, M. J. Hayman, and P. K. Vogt.** 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. Cell **11:**95–103.
34. **Norton, P. A., and J. M. Coffin.** 1987. Characterization of Rous sarcoma virus sequences essential for viral gene expression. J. Virol. **61:**1171–1179.
35. **Payne, L. N., and H. G. Purchase.** 1991. Leukosis/sarcoma group, p. 386–439. In B. W. Calnek (ed.), Diseases of Poultry, 9th ed. Iowa State University Press, Ames.
36. **Resnick, R. M., M. T. Boyce-Jacino, Q. Fu, and A. J. Faras.** 1990. Phylogenetic distribution of the novel avian endogenous provirus family EAV-0. J. Virol. **64:**4640–4653.
37. **Robertson, J. S., C. Nicolson, A. M. Riley, M. Bentley, G. Dunn, T. Corcoran, G. C. Schild, and P. Minor.** 1997. Assessing the significance of reverse transcriptase activity in chick cell-derived vaccines. Biologicals **25:**403–414.
38. **Smith, R. E.** 1982. Avian osteopetrosis. Curr. Top. Microbiol. Immunol. **101:**75–94.
39. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
40. **Tereba, A., and S. M. Astrin.** 1980. Chromosomal localization of *ev-1*, a frequently occuring endogenous retrovirus locus in White Leghorn chickens, by in situ hybridization. J. Virol. **35:**888–894.
41. **Tsang, S. X., W. M. Switzer, V. Shanmugam, J. A. Johnson, C. Goldsmith, A. Wright, A. Fadly, D. Thea, H. Jaffe, T. M. Folks, and W. Heneine.** 1999. Evidence of avian leukosis virus subgroup E and endogenous avian virus in measles and mumps vaccines derived from chicken cells: Investigation of transmission to vaccine recipients. J. Virol. **73:**5843–5851.
42. **Vogt, P. K., J. L. Spencer, W. Okazaki, R. L. Witter, and L. B. Crittenden.** 1977. Phenotypic mixing between reticuloendotheliosis virus and avian sarcoma viruses. Virology **80:**127–135.
43. **Weber, F., and W. Schaffner.** 1985. Enhancer activity correlates with the oncogenic potential of avian retroviruses. EMBO J. **4:**949–956.
44. **Weissmahr, R. N., J. Schüpbach, and J. Böni.** 1997. Reverse transcriptase activity in chicken embryo fibroblast culture supernatants is associated with particles containing endogenous avian retrovirus EAV-0 RNA. J. Virol. **71:**3005–3012.
45. **World Health Organization Expert Committee on Biological Standardization.** 1994. Requirements for measles, mumps and rubella vaccines and combined vaccines (live). Requirements for biological substances, no. 47. WHO Tech. Rep. Ser. **840:**100–207.