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.

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 (CEF) 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 sources. CEF is susceptible 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.

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 non-neoplastic 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\(^{2}\) 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\(^{2}\) 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)
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 interstrain 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-7 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\(_{13}\)) 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\% trypotype 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 virus vaccines. Japanese quail fibroswcaroma 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.

**Long-template PCR amplification of ev loci and genomic RNA.** To analyze ev loci by ev 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 (2 × 10\(^5\) 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 8.0], 0.5% 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 of (0.25 \(\mu\)g) 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, ev-3, ev-6, and ev-21 loci; 54°C for ev-2, ev-3, ev-7, and ev-9; 56°C for ev-6; and 60°C for ev-12; 52°C for ev-7; and 53°C for ev-9 and ev-21.

**Long-template PCR amplification of ev loci and genomic RNA.** To analyze ev-1 and ev-3, specific amplification of each locus was performed by long-template PCRs (Boehringer-Mannheim). Primers evIlocF2 (modified from reference 1) and evIlocF1 (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 5' untranslated region primer (ev_3UTRR1) (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 evIllocR2 and ev3locR2 were used in conjunction with evpolF8 to amplify the sequence from pol to the

### Table 1. Primers used in this study for ev loci and genomic RNA

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAVLTRdF</td>
<td>TTGTAATAGGCGTATGCAGGTT</td>
</tr>
<tr>
<td>EAVLTRdR</td>
<td>TGCTCAAGGAGCTTATCAGAGGAAATGAGCT</td>
</tr>
<tr>
<td>evLTR2</td>
<td>CTGCTCAGCTTATCAGAGGAAATGAGCT</td>
</tr>
<tr>
<td>evLTRd2</td>
<td>GTGATCCAGCGCTTATCAGAGGAAATGAGCT</td>
</tr>
<tr>
<td>evlocF2</td>
<td>GGCAACAAAAATCTAGATCGTGCATG</td>
</tr>
<tr>
<td>evlocR2</td>
<td>ATTCAGGAGCTTATCAGAGGAAATGAGCT</td>
</tr>
<tr>
<td>ev3polF1</td>
<td>TCTCGAGCGAGCTTATCAGAGGAAATGAGCT</td>
</tr>
<tr>
<td>ev3polR1</td>
<td>TTTTGGGAGATATGTCTCTGATACATA</td>
</tr>
<tr>
<td>ev3polF2</td>
<td>CACCCATCTCGTGCATGCTGATACATA</td>
</tr>
<tr>
<td>ev3polR2</td>
<td>AGACTGGCCACCGATGGGCAGT</td>
</tr>
<tr>
<td>ev3polF3</td>
<td>TGGTGGCGACTAGATAAGGAAGAT</td>
</tr>
<tr>
<td>ev3polR4</td>
<td>TGGTGGCGACTAGATAAGGAAGAT</td>
</tr>
<tr>
<td>ev3polF5</td>
<td>CCTGATGTTGCTAGACCTAAGAT</td>
</tr>
<tr>
<td>ev3polR5</td>
<td>CAGAGGAGGCGAGGCTAATAA</td>
</tr>
<tr>
<td>evpolF8</td>
<td>GCCGATCCCTTGTGAGAGGGCTAAG</td>
</tr>
<tr>
<td>evpolR3</td>
<td>CTTTGAGGAGCTTATCAGAGGAAATGAGCT</td>
</tr>
<tr>
<td>evpolresREV</td>
<td>ACCCTTGCAGAAATAGGGCAAAGGAGT</td>
</tr>
</tbody>
</table>
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 evLTRA2 reverse primer, which annealed to the 3' end of the ev-6 locus. The forward primers espF2, ev_5polF2, and espF4 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 x g for 10 min. Virus particles were harvested from 100 ml of clarified culture supernatant at 100,000 x 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 ev3R2, which flank the deletion in ev-3 and yield a 761-bp fragment that is diagnostic of ev-3. The evFL3 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 μl 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 32P-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 espF2 and espF4 and evpolREV, which yielded a 3,139-bp fragment. A 1,909-bp fragment, which included the signature mutation of ev-1 as well as other sequence changes for comparison, was subsequently amplified with the nested primers ev6spF2 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 WQ G4. 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 x 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 then were 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.

![FIG. 1. Schematic of ev loci and regions of primer hybridization used in the detection and amplification of ev sequences.](image)

### TABLE 2. ev probes used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAVLTrd.1P</td>
<td>GTACACGGAGGGTTAAAGAGATAAT ev gag-pol.1P ...............................................</td>
</tr>
<tr>
<td>evmidpol.1P</td>
<td>TTTGTTGCGAAAATGTTACTCAAG ev p19.2P ......................................................</td>
</tr>
<tr>
<td>evLTRA.1P</td>
<td>TAGAACGAACTGTTACGATGTTAAGATATA ev polF2 ................................................</td>
</tr>
<tr>
<td>evspenv.1P</td>
<td>CTAATACTACTAGATTTCCTAGT ev FLF3 ............................................................</td>
</tr>
<tr>
<td>evpolR3</td>
<td>TAGACGAACTGTTACGATGTTAAGATATA evpolF4 ..................................................</td>
</tr>
<tr>
<td>evpolREV</td>
<td>CTAATACTACTAGATTTCCTAGT evFLR3 ...............................................................</td>
</tr>
<tr>
<td>evLTRA.1P</td>
<td>TAGACGAACTGTTACGATGTTAAGATATA evFLR3 .....................................................</td>
</tr>
<tr>
<td>evspenv.1P</td>
<td>CTAATACTACTAGATTTCCTAGT evpolREV ............................................................</td>
</tr>
<tr>
<td>evpolR3</td>
<td>TAGACGAACTGTTACGATGTTAAGATATA evFLR3 .....................................................</td>
</tr>
<tr>
<td>evLTRA.1P</td>
<td>TAGACGAACTGTTACGATGTTAAGATATA evFLR3 .....................................................</td>
</tr>
</tbody>
</table>
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 evLTRd2P probe (Table 2). Additionally, all samples were screened for two chicken DNA sequences to exclude contamination by residual CEF DNA. Primers evLTRF1 and evLTRcR2, based on the ev-1 LTR and flanking chicken sequence, were used to screen for the ubiquitous ev-1 locus-specific sequence. Additionally, the EAVLTRdF1 and EAVLTRdR1 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 evLTRdF1 and EAVLTRdR1 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 \(\mu\)l 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 expolF3 and expolnesREV primers were used in a primary RT-PCR, and the sequence of interest was amplified by nested PCR using the expolF5 and expolR3 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 \(\mu\)Ci of \([\gamma-\text{32P}]\)ATP in a total volume of 50 \(\mu\)l. Probes were purified on Sephadex G-50 spin columns (Pharmacia Biotech) to remove unbound \([\gamma-\text{32P}]\)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 \(\mu\)Ci of \([\alpha-\text{32P}]\)CTP 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.

RESULTS

RFLP analysis of CEF genomic DNA. Southern blot hybridization of BamHI-digested CEF DNA from the WG Qual. 4 vaccine substrate lot with the expol-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 SacI digested 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 BamHI digest were corroborated by the restriction pattern of the SacI 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 adenine 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 AY013303, and AY013304, respectively).
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 ev1spR1 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 (GTTTATATAAACATATAAA), not homologous to ALV-E, is present at the site of the deletion and may be the remnant of a recombination 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 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 4024 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% homology 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 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-like sequences, as well as the orf and flanking 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.
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.

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 poly(A) 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 MMRII 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 Althal Hussain for performing the Amp-RT analysis, and to Thomas M. Folks and Alison Mawle for critical review of the manuscript.

REFERENCES


