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# Reverse Transcriptase Activity in Chicken Embryo Fibroblast Culture Supernatants Is Associated with Particles Containing Endogenous Avian Retrovirus EAV-0 RNA

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**We have recently shown that live attenuated virus vaccines produced on chicken-derived cells contain low levels of particle-associated reverse transcriptase (RT). In both virus and corresponding control harvests produced on chicken embryo fibroblasts, these activities were present at significantly higher concentrations than in the vaccines. In order to identify the putative retrovirus sequence responsible for this activity, a novel method for the selective PCR amplification of particle-associated retrovirus RNA that uses DNA primers complementary to the primer binding sites of the known exogenous retroviruses in combination with an anchor primer was applied. A product of the endogenous avian retrovirus family EAV-0, termed EAV-$0_{B1}$, was reproducibly generated with a $tRNA^{Trp}$-derived primer from the RT peak fraction of a sucrose density gradient run with a harvest of a live attenuated measles vaccine. In contrast, no products were detected with primers derived from $tRNA^{Pro}$, $tRNA^{Lys}_{1,2}$ or $tRNA^{Lys}_{3}$. In the same fraction, genomic RNA of EAV-$0_{B1}$ was demonstrated by long PCR. Analysis of several sucrose density gradients from different harvests of various manufacturers demonstrated accumulation of, and colocalization with, RT activity for the EAV-$0_{B1}$ RNA but not for a chicken cellular mRNA. Synthesis of cDNA from EAV-$0_{B1}$ RNA was shown by endogenous RT reaction. Furthermore, complexes of naturally primed EAV-$0_{B1}$ RNA with RT were demonstrated. Taken together, these data strongly suggest that EAV-0 is able to produce virus-like particles with an active RT.**

Live attenuated virus vaccines are successfully used worldwide. Their contribution in preventing morbidity and mortality is evident. However, the presence of undesired microorganisms or other transmissible agents is a risk factor, since they cannot be subjected to inactivation procedures and because most of them are administered by parenteral injection, thus bypassing biochemical and physical barriers such as gastric acid or the skin. In order to avoid the release of contaminated vaccines, intensive testing for a wide variety of specific pathogens is required (24).

We recently reported the systematic presence of low levels of particle-associated reverse transcriptase (RT) in live attenuated virus vaccines that are produced in cells of chicken origin (7). RT activity was detected by an ultrasensitive RT test, named product-enhanced RT (PERT) assay, which has a detection limit that is $10^6$ to $10^7$ times lower than that of conventional RT tests (18) and is at least equivalent in sensitivity to RNA-PCR (6). The activity in all chicken-derived vaccines was shown to be inhibited by zidovudine (AZT)-triphosphate and ddTTP, i.e., agents known to specifically inhibit retrovirus reverse transcriptases. Furthermore, sucrose density banding, ultracentrifugation, and filtration experiments suggested that RT activity was associated with particles which banded at a density compatible with that of retroviruses (7). The presence of RT activity in these vaccines was quickly confirmed by several other laboratories using related tests (25).

Based on the findings discussed above, we hypothesized that some kind of retrovirus particles might be present in chicken cell-derived vaccines. Since these vaccines are regularly tested for exogenous viruses and because the activity was present in all chicken cell-derived vaccines independent of manufacturer, lot, or production year, an endogenous retrovirus was more likely than an exogenous one. In order to identify this putative virus, an efficient strategy for a selective amplification of particle-associated retrovirus RNA (PARRA) was applied. This procedure identifies 5′ R-U5 and subsequently 3′ R sequences of retrovirus particles, even at concentrations as low as 1,000 particles/ml (22).

In this study, we report the identification, in harvests of live attenuated virus vaccines, of a retrovirus RNA which belongs to the EAV-0 family and is enclosed in particles which contain RT. EAV-0 elements belong to a group of endogenous avian retroviruses which have been isolated from the genomic DNA of line 0 chickens, a strain lacking classical subgroup E endogenous virus loci ($ev^-$) (12). Our findings suggest not only that EAV-0 proviruses are transcribed but also that genomic RNA is packaged into retrovirus-like particles containing a functional RT.

## MATERIALS AND METHODS

**Virus harvests and cell cultures.** Virus harvests of live attenuated measles and mumps virus were obtained from the manufacturers through the Swiss Federal Office of Public Health. These harvests were collected from primary chicken embryo fibroblast (CEF) cultures which were produced from 11-day-old chicken embryos of specific-pathogen-free flocks and inoculated with seed virus. The codes used for the different manufacturers are the same as those previously published (7). Aliquots were stored at −70°C until use. MRC-5, a human diploid lung cell line, was obtained from the American Type Culture Collection, Rockville Md. (CCL-171). The cloned human T-cell leukemia virus type 2 (HTLV-2) producer cell line 76D9 was from J. Jendis (our laboratory).

**Sucrose gradient density banding.** Virus harvest fluid (3 ml) precleared at 16,000 × *g* for 10 min, was pelleted at 70,000 × *g* for 90 min, resuspended in 100 μl of phosphate-buffered saline, and layered on a sucrose gradient (7.5 to 60%). The gradient was ultracentrifugated at 30,000 × *g* for 16 h at 4°C in a Kontron TFT32.13 rotor; fractions of 400 μl were manually collected from the top, and their densities were determined in a Zeiss refractometer. Five microliters of each fraction was analyzed for $Mg^{2+}$-dependent RT activity by PERT assay.

**PERT assay.** The PERT assay, a test for the detection of extremely low levels of RT activity, was performed as described elsewhere (7).

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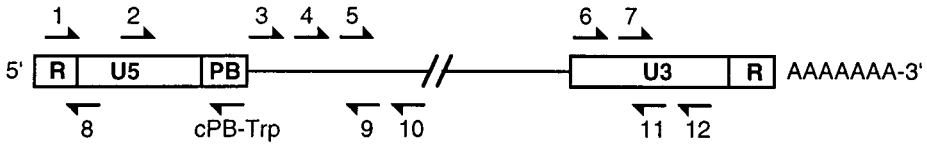


FIG. 1. Relative location and orientation of the EAV-0 specific oligonucleotide primers. The arrows indicate primers no. 1 to 12 and the cPB-Trp primer set.

**PARRA. (i) cDNA synthesis.** cDNA synthesis was performed in a final volume of 15 μl containing 6 μl of sucrose gradient peak fraction; 50 μg of Dynabeads Oligo $(dT)_{25}$ (DYNAL); 10 pmol of primer cPB-Trp-10, cPB-Lys1,2-10, cPB-Lys3-10, or cPB-Pro-10; 0.5 mM (each) deoxynucleoside triphosphate (dNTP); 0.25% Nonidet P-40 (NP-40); 15 U of RNase inhibitor (rRNasin; Promega); 200 U of SuperScript II RNaseH$^-$ RT (Gibco BRL); and 1× cDNA synthesis buffer (Gibco BRL). The reaction was carried out at 37°C for 60 min.

**(ii) Tailing of cDNA.** Prior to tailing, the beads were washed with wash buffer (20 mM Tris HCl [pH 8], 50 mM KCl), and then 25% of the cDNA-RNA hybrids were subjected to RNase H degradation. Degradation was performed in a volume of 5 μl containing 10 mM Tris HCl (pH 7.5), 3 mM $MgCl_2$, 50 mM KCl, 10 mM dithiothreitol (DTT), 0.005% NP-40, and 0.3 U of RNase H (Boehringer Mannheim). The reaction was carried out at 37°C for 15 min followed by 65°C for 5 min. A volume of 5 μl containing 10 mM Tris HCl (pH 9), 50 mM KCl, 200 μM dCTP, and 15 U of terminal deoxynucleotidyl transferase (Gibco BRL) was added. The mixture was incubated at 37°C for 20 min followed by inactivation of the enzyme at 65°C for 5 min.

**(iii) Anchored PCR of the 5′ terminal region.** Two successive rounds of PCR were performed with the Expand Long Template PCR System kit (Boehringer Mannheim). The first round started with a total volume of 10 μl containing 1 μl of tailing reaction mixture and 0.2 pmol of anchor primer BESII-40G. Three initial cycles of 94°C for 30 s, 60°C for 1 min, and 68°C for 1 min (PHC-2 cycler; Techne) were performed and then a PCR mixture containing 20 pmol of primer BESII-40G and 40 pmol of primer cPB-Trp-16, cPB-Lys1,2-13, cPB-Lys3-13, or cPB-Pro-13 was added to a final volume of 50 μl, followed by 35 cycles of 94°C for 30 s, 50°C for 1 min, and 68°C for 1 min. One microliter from the first amplification mixture was subjected to a second seminested PCR consisting of 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, with 20 pmol of primer BESII-26G; 40 pmol of primer cPB-Trp-19, cPB-Lys1,2-18, cPB-Lys3-16, or cPB-Pro-19; and 1.25 U of *Taq* DNA polymerase (Perkin-Elmer).

**(iv) Anchored PCR of the 3′ terminal region.** Two successive rounds of PCR were performed. The first PCR round, which contained 1/15 of cDNA bound to Dynabeads from the cDNA reaction described above (see "cDNA synthesis"), 40 pmol of primer no. 6, and 1.25 U of *Taq* DNA polymerase, started with three initial cycles of 94°C for 30 s, 65°C for 1 min, and 68°C for 1 min. Then, 20 pmol of anchor primer BESII-40T was added to a final volume of 50 μl, followed by an additional 40 cycles of 94°C for 30 s, 50°C for 1 min, and 68°C for 1 min. One microliter from the first amplification mixture was subjected to a second seminested round for 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, with 40 pmol of nested primer no. 7 and 20 pmol of primer BESII-26G.

**Sequencing.** PCR products were isolated from low-melting-point gel by agarase digestion (Boehringer Mannheim) according to the manufacturer's instructions and sequenced by automated cycle sequencing on an ABI 373A DNA sequencer (Perkin-Elmer). Sequencing primers were cPB-Trp-19 for the 5′ product and primer no. 7 for the 3′ anchored PCR product.

**Long PCR of viral cDNA.** The first round of long PCR was performed according to the manufacturer's instructions for the Expand Long Template PCR kit (Boehringer Mannheim) with the pair of primers no. 12 and no. 3 and 1/15 of viral cDNA bound to Dynabeads from the cDNA reaction described above (see "cDNA synthesis"). The second round of long PCR was performed with the pair of primers no. 4 and no. 11 and 2% of the first long PCR mixture.

To demonstrate that no cellular EAV-0 DNA was bound to the beads, 1/30 of the cDNA reaction mixture was subjected to PCR for 40 cycles of 94°C for 30 s, 65°C for 1 min, and 72°C for 1 min with primers no. 6 and no. 10.

**RNA amplification of EAV-0, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and HTLV-2. (i) RNA hybridization.** RNA from 10 μl of each sucrose gradient fraction was hybridized together with an aliquot of HTLV-2 containing culture supernatant for 30 min at room temperature to 50 μg of Dynabeads Oligo $(dT)_{25}$ in a final volume of 40 μl containing 50 mM HEPES (pH 7.5), 250 mM NaCl, 50 ng of bovine serum albumin, 10 mM DTT, 0.5% NP-40, and 40 U of rRNasin. RNA bound to the beads was washed with 1× cDNA synthesis buffer.

**(ii) cDNA synthesis.** RNA bound to the beads was subjected to cDNA synthesis in a final volume of 20 μl, with the reagents and reaction conditions described above. cDNA bound to the beads was washed with 1× PCR buffer.

**(iii) 3′ anchored PCR.** For EAV-0 and HTLV-2 detection, 2 μl from the cDNA synthesis reaction mixture was subjected to 3′ anchored PCR with 1.25 U of *Taq* DNA polymerase and primers BESII40-TG, no. 6, and HT-1. For chicken GAPDH and HTLV-2 detection, 2 μl from the cDNA synthesis reaction mixture was subjected to 3′ anchored PCR with primers BESII40-TG, BESII40-T, CHIGA-5, and HT-1. The first cycle was performed at 94°C for 15 s, 60°C for 1 min, and 72°C for 1 min, followed by 35 cycles of 94°C for 15 s, 55°C for 1 min, and 72°C for 1 min on a Progene Cycler (Techne).

**Southern blotting of amplified cDNA.** Amplified cDNA (15 μl) was separated on a 2% agarose gel and blotted to a nylon membrane (Hybond-N plus; Amersham) by alkaline transfer, according to the manufacturer's protocols. Probes were labeled with [α-$^{32}$P]dCTP as previously described (20). For EAV-0, probes no. 7 and no. 11 were used for 3′ anchored PCR [U3-R-poly(A)]; no. 2 and no. 2a were used for R-U5 PCR; and no. 5 and no. 9 were used for *gag*-leader PCR. For chicken GAPDH, probes CHIGA-6 and CHIGA-6a were used for 3′ anchored PCR. For HTLV-2, probes HT-2 and HT-2a were used for 3′ anchored PCR. Amplification products were visualized on a PhosphorImager (Molecular Dynamics) and printed by the Adobe Photoshop program (version 2.0.1; Macintosh).

**Endogenous RT reaction.** Reaction mixtures containing 2 μl of sucrose gradient fraction no. 10; 1× cDNA synthesis buffer (Gibco BRL); 10 mM DTT; and 0%, 0.01%, or 0.4% NP-40, were preincubated at room temperature for 10 min prior to the addition of 0.5 mM dNTPs and 20 pmol of $dT_{15}$ (Boehringer Mannheim) to a final volume of 30 μl. Inhibition of endogenous RT reaction was performed with the addition of 0.075 mM AZT-triphosphate. For the positive control, 20 U of SuperScript II RNaseH$^-$ RT (Gibco BRL) was added. The reaction mixtures were incubated at 37°C for 15 min, and the reactions were terminated by incubation at 75°C for 5 min. Five microliters of the endogenous RT reaction was subjected to PCR with 40 pmol of primers no. 6 and BESII-40TG, with a first cycle of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min and a second cycle of 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min.

**Association of RT with RNA template.** RNA from 5 μl of sucrose gradient fraction no. 10 was hybridized for 30 min at room temperature to 50 μg of Dynabeads Oligo $(dT)_{25}$ in a final volume of 10 μl containing 1× cDNA synthesis buffer, 10 mM DTT, and 0.5% NP-40. RNA bound to the beads was washed with 1× cDNA synthesis buffer and incubated for 60 min in a volume of 20 μl containing 1× cDNA synthesis buffer, 10 mM DTT, 0.005% NP-40, and either 0.5 mM dNTPs, no dNTPs, or 0.5 mM dNTPs with 20 U of SuperScript II RNaseH$^-$ RT. cDNA bound to the beads was washed with 1× PCR buffer. Twenty-five percent of the washed beads were subjected to PCR with 40 cycles as described above for the endogenous RT reaction. Primers no. 1 and cPB-Trp-19 were used for 5′ R-U5 PCR, primers no. 6 and BESII-40TG were used for 3′ U3-R-poly(A) anchored PCR, primers no. 6 and no. 8 were used for U3-R-U5 PCR, and primers no. 3 and no. 10 were used for *gag*-leader PCR.

**Primer sequences.** The primers used and their sequences are as follows: cPB-Trp-10 (5′-AATCACGTCG-3′), cPB-Trp-16 (5′-AATCACGTCGGGGTCA-3′), cPB-Trp-19 (5′-AATCACGTCGGGGTCACCA 3′), cPB-Lys1,2-10 (5′-GCCCCACGTT-3′), cPB-Lys1,2-13 (5′-GCCCCACGTTGGG-3′), cPB-Lys1,2-18 (5′-GCCCCACGTTGGGCGCCA-3′), cPB-Lys3-10 (5′-AGTCCCTGTT-3′), cPB-Lys3-13 (5′-GTCCCTGTTCGGG-3′), cPB-Lys3-16 (5′-AGTCCCTGTTCGGGCG-3′), cPB-Pro-10 (5′-AATCCCGGAC-3′), cPB-Pro-13 (5′-AATCCCGGACGAG-3′), cPB-Pro-19 (5′-AATCCCGGACGAGCCCCCA-3′), BESII-40G (5′-CGCGGTGATCAGAATTCGTCGACAGGGGGGGGGGGGGGG-3′), BESII-26G (5′-CGCGGTGATCAGAATTCGTCGACAGG-3′), BESII-40T (5′-GAATTCGTCGACAGGTTTTTTTTTTTTTTTTTTTTTTTT-3′), BESII-40TG (5′-GAATTCGTCGACAGGTTTTTTTTTTTTTTTTTTTTTTTTG-3′), no. 1 (5′-GCCATTTTGCCGCTCATCATATTGGTGTCA-3′), no. 2 (5′-CAGGCTCCCCTAAGCAACGAACATCACGG-3′), no. 2a (5′-GGCAACCGTGATGTT-3′), no. 3 (5′-GCTTAGGGGAATGAGTCGGCTGTCTGCTTG-3′), no. 4 (5′-GGCTGTCTGCTTGACGGGGCAGGAG-3′), no. 5 (5′-GGACAGAGGGAATCTG-3′), no. 6 (5′-ATAGGCGTGATCGGGGTCTCGGGATG-3′), no. 7 (5′-CGTGTCAGGCTCCTCCCCATGTGTTAGG-3′), no. 8 (5′-GCCAAATACCGAGGTGACACCAATATGATGAG-3′), no. 9 (5′-GGTGACTGCAGATTCC-3′), no. 10 (5′-GTCCGCGACCTCCGCCGACAGAG-3′), no. 11 (5′-CGTGGCACGTACCTAACACATGGGGAG-3′), no. 12 (5′-CCCTTCGTGTACGCCCACTGGATGG-3′), HT-1 (5′-AGCGCAAGGACAGTTCAGGAGG-3′), HT-2 (5′-GAGGTTTCAAGCCAGAGTCTAGCCGAG-3′), HT-2a (5′-GTGCTCGGCTAGA-3′), CHIGA-5 (5′-GGAAGCAGGACCCTTTGTTGGAG-3′), CHIGA-6 (5′-GGTGAACACTTTTATTGATGTAAGGTGG-3′), and CHIGA-6a (5′-GAGCCACCTTACA-3′). The design of primers no. 1 to 12 was based on the EAV-$0_{B1}$ sequence and EAV-0 sequence from GenBank (accession number M31066). The relative localization of the EAV-0-specific primers is shown in Fig. 1. HT-1, HT-2, and HT-2a primers are based on the HTLV-2 sequence from GenBank (accession number L20734). CHIGA-5, CHIGA-6, and CHIGA-6a are based on the chicken GAPDH sequence from GenBank (accession number K01458).

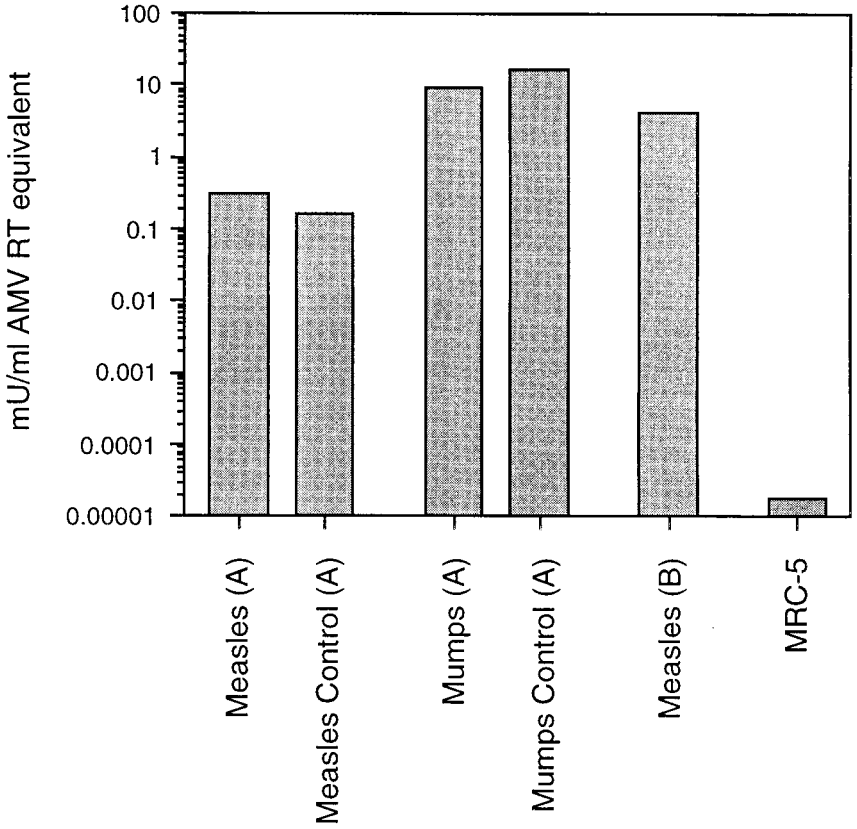


FIG. 2. RT activity in harvests of live attenuated virus vaccines determined by PERT assay. The bars show RT activity in harvests containing live attenuated measles or mumps virus in comparison with supernatants from controls of the same lot of cells, which had not been inoculated with vaccine virus. All harvests were supernatants of primary CEF cultures from manufacturer A or B (7). The human MRC-5 cell line was used as a negative control. A half-logarithmic dilution series of purified avian myeloblastosis virus (AMV) RT (Boehringer Mannheim) was used as an external standard for quantification (7).

## RESULTS

**RT activity is present in all virus vaccine harvests of chicken cell origin, independent of vaccine virus.** It has been demonstrated that live attenuated virus vaccines produced in chicken cells contain RT activity (7). The starting material for vaccine production is the so-called harvest, which is either a supernatant of CEF cultures or allantoic fluid of embryonated eggs which were infected with seed virus. CEF harvests from two manufacturers which contained live attenuated measles or mumps virus were tested for RT activity by the PERT assay (Fig. 2). RT activity was found to be independent of manufacturer and vaccine virus. CEF control harvests, i.e., culture supernatants from uninoculated CEFs of the same lot, showed comparable levels of RT activity, indicating that RT activity was neither induced nor introduced by the vaccine virus. These data are consistent with an endogenous retrovirus being the cause of the RT activity. As expected, activity in the harvests was higher than that in the final diluted vaccine product (7). Interestingly, RT activity in different control harvests from the same manufacturer varied by up to two orders of magnitude.

**Amplification and sequencing of particle-associated retrovirus RNA revealed an EAV-0 sequence.** To prove the hypothesis that the detected RT activity originated from a retrovirus, an efficient strategy for a selective PARRA was applied. The PARRA method was designed to amplify RNA sequences of small numbers of retrovirus particles, for which no specific sequence information other than the 18 nucleotides of the known tRNA primer binding sites (PBs) is available (22). The PARRA strategy is illustrated in Fig. 3. An aliquot containing a presumed retrovirus is subjected to cDNA synthesis primed both at the 3′ end of the viral RNA with oligo(dT) magnetic beads and at the 5′ terminal region with a sequence-specific primer complementary to the retrovirus PB (cPB primer) (Fig. 3). Since most retroviruses discovered to date have one of only four different PBs complementary to the 3′ end of $tRNA^{Pro}$, $tRNA_3^{Lys}$, $tRNA_{1,2}^{Lys}$, or $tRNA^{Trp}$, four cDNA synthesis reactions for each retrovirus candidate have to be performed with the corresponding cPB primers. The generated cDNA is elongated with a homopolymeric tail and subsequently amplified by anchored PCR (14, 16) of the 5′ terminal region. The resulting 5′ R-U5 PCR product is directly sequenced. If the sequence at hand is of retroviral origin, the R-poly(A) region of the viral 3′ end will bind to the oligo(dT) beads. To verify the presence of the R-poly(A) sequence, anchored PCR of the 3′ terminal region is performed. Since the sequenced R region is present at each end of the retrovirus RNA genome, a sequence-specific primer located in the R region can be used to amplify the R-poly(A) region, which can subsequently be sequenced.

Presumed retrovirus particles from the harvest of live attenuated measles vaccine (manufacturer B) (7) were sedimented by ultracentrifugation, resuspended, and banded by sucrose gradient density banding. An aliquot of the sucrose gradient RT peak fraction of measles virus harvest was subjected to PARRA, which resulted in a predominant 5′ amplification product of 160 bp when cPB-Trp primers were used. Repli-

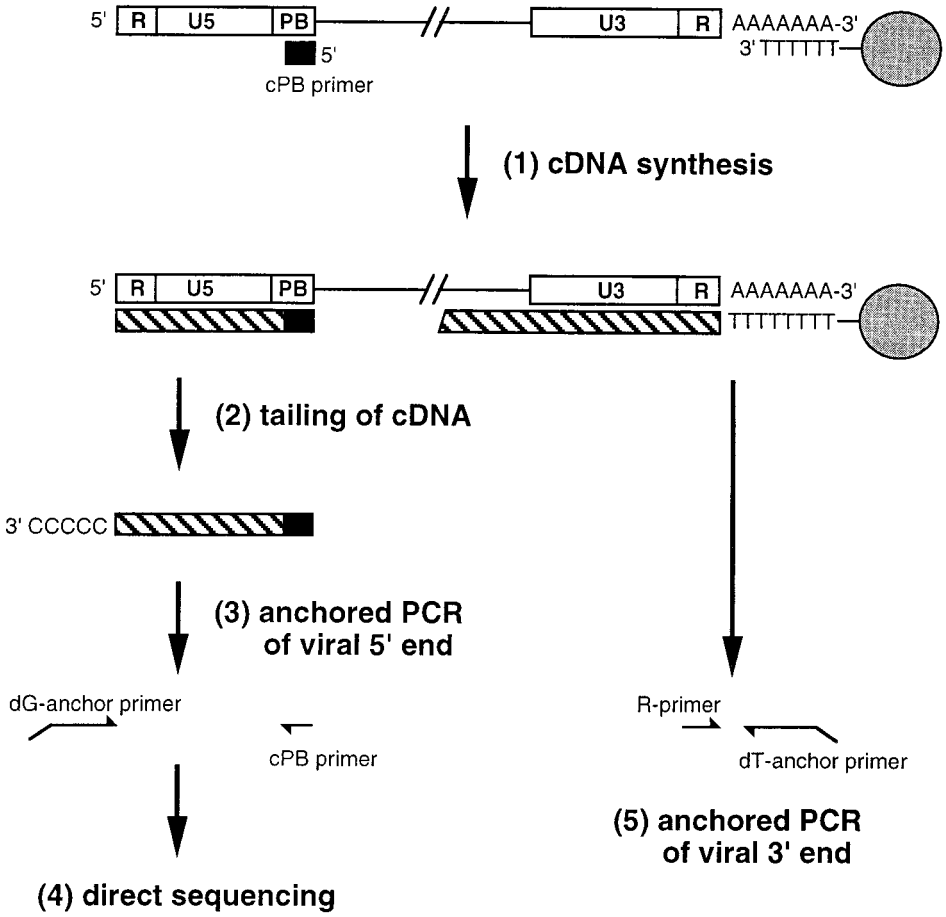


FIG. 3. Strategy to detect and identify particle-associated retrovirus RNA. The solid box represents the DNA oligonucleotide primer complementary to the PB (cPB). The shaded circle symbolizes an oligo(dT) magnetic bead. The hatched boxes represent cDNA. Following the sequence identification of the 5′ R-U5 region (steps 1 to 4), an R-specific primer is designed to perform 3′ anchored PCR (step 5) for the identification of the 3′ end.

cates of 5′ terminal amplification confirmed this product (Fig. 4, lanes 1 to 4), whereas no products were obtained with primer cPB-Lys1,2 (lanes 5 to 8), cPB-Lys3 (lanes 9 to 12), or cPB-Pro (lanes 13 to 16). Sequencing and a subsequent similarity search of all nucleic acid sequences in the National Center for Biotechnology Information Entrez data bank (release 22) revealed identity with an R-U5 sequence belonging to the EAV-0 family. Sequencing showed that none of the other products visible in lanes 1 to 4 (Fig. 4) was related to retrovirus elements.

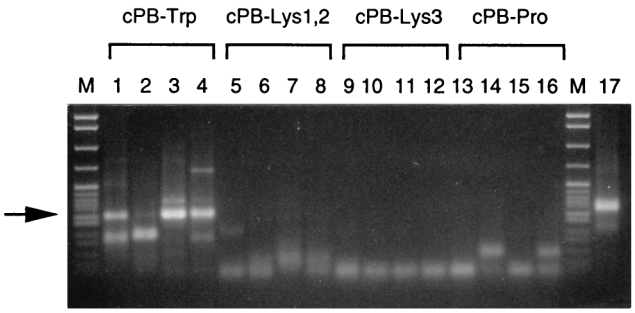


FIG. 4. Agarose gel with amplification products of the PARRA strategy. RNA was reverse transcribed and tailed as shown in Fig. 3, and 5′ R-U5 replicates were amplified with the anchor primer and primers complementary to the tryptophan (cPB-Trp; lanes 1 to 4), lysine 1,2 (cPB-Lys1,2; lanes 5 to 8), lysine 3 (cPB-Lys3; lanes 9 to 12), or proline (cPB-Pro; lanes 13 to 16) PB. The arrow marks a repeatedly present amplification product of approximately 160 bp in length. The band was excised, sequenced, and designated EAV-$0_{B1}$. Lane 17, anchored PCR of the 3′ end starting from the same cDNA synthesis reaction mixture as in lanes 1 to 4. M, molecular size marker pBR322 DNA-*Msp*I digest (New England Biolabs) (for identification of the size, see Fig. 6B).

To demonstrate the corresponding 3′ end of this RNA sequence, which was named EAV-$0_{B1}$, anchored PCR of the 3′ terminal region was performed. Since the predicted R region of EAV-0 is short, primers within the EAV-0 U3 region were designed for amplification based on the published U3 region (9). An amplification product with a predicted length of 175 bp was obtained (Fig. 4, lane 17). Sequencing verified that the product was a U3-R sequence with a poly(A) tail. Comparison of the 5′ and 3′ R sequences showed that they were identical (Fig. 5). The R region was found to have a length of 19 bp, and the U5 region measured 80 bp, contrary to the findings of Boyce-Jacino et al., who speculated that R had a length of 21 bp and U5 had a length of 81 bp (9).

In order to demonstrate the presence of full-length RNA (8), the entire genomic cDNA bound to the beads was amplified by long PCR (2, 10, 11). This resulted in a 5.8-kb product (Fig. 6A), which was confirmed to be EAV-0 by molecular cloning and sequencing (23). PCR of the U3-R-U5 long terminal repeat region, a structure found only in proviral DNA, revealed no product (Fig. 6B). This negative result thus strongly suggests that the long PCR product originated from cDNA.

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                <=        R       =><=                    U5
5'R-U5:  1   GCCATTTTGC CGCTCATCAT ATTGGTGTCA CCTCGGTATT TGGCCAAGCC GCAGGCTCCC
                                                      =>  <= Trp-PB
        61   CTAAGCAACG AACATCACGG TTGCCTGCGA AAGGCAACAAG


3'U3-R:  1   TACGTGCCAC GTGTACCATC CAGTGGGCGT ACACGAAGGG TTAAAAGATA TATAAGTGCT
                    U3        =><=         R       =><= poly(A)
        61   TGTTAGAACT TAATAAACGC CATTTTGCCG CTCATCA
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FIG. 5. Nucleotide sequences of EAV-$0_{B1}$. (Top) Complete 5′ R-U5 sequence obtained by 5′ anchored PCR. (Bottom) 3′ U3-R sequence obtained by 3′ anchored PCR with primer no. 7 and the dT anchor primer. Position 1 corresponds to the first nucleotide downstream of primer no. 7 within the U3 region. The R region, whose 3′ end is defined by the site of the poly(A) tail addition, has a length of 19 bp. The U5 region is 80 bp long. The sequences are identical with the published EAV-0 proviral clone g17.5 (GenBank accession number M31066). Trp-PB, tryptophan PB.

**Colocalization of RT activity and EAV-0 RNA peak.** If virus-like particles containing RT were indeed encoded by EAV-$0_{B1}$, a colocalization of EAV-$0_{B1}$ RNA and RT activity would be expected. Sucrose gradient density banding was performed with undiluted measles virus harvest from manufacturer B, and each fraction of the gradient was analyzed by PERT assay. $Mg^{2+}$-dependent RT activity peaked at a density of 1.13 g/ml (Fig. 7A). This is below 1.14 g/ml, the density at which retrovirus particles typically band under the conditions used in our laboratory (18). Similar results were obtained with measles harvest from manufacturer A (data not shown).

Since the gradient was considerably contaminated with cellular EAV-0 DNA, a procedure to specifically amplify RNA was performed. Each sucrose gradient fraction was subjected to cDNA synthesis primed by oligo(dT) magnetic beads followed by anchored PCR of the retrovirus 3′ terminal region. To each reaction mixture, a predetermined amount of HTLV-2 particles was added as an internal control. The EAV-$0_{B1}$ RNA accumulated in a peak which colocalized with the RT activity peak, while the signals generated from the HTLV-2 RNA were even with all fractions. This showed that the EAV-0 product accumulation in fractions no. 6 to 12 was not due to an artifact (Fig. 7B). As expected, no product was obtained by amplification of the U3-R-U5 long terminal repeat, which indicated that only RNA transcripts, but no cellular EAV-0 DNA, were bound to the beads (data not shown). To further demonstrate that the accumulation of EAV-$0_{B1}$ RNA at a density of 1.13 g/ml was selective, an aliquot of the cDNA synthesized as described above was subjected to anchored PCR of the 3′ terminal region of chicken GAPDH mRNA. In contrast to EAV-$0_{B1}$ RNA, cellular GAPDH mRNA was evenly distributed over the entire sucrose gradient (Fig. 7C). Accumulation and colocalization of the EAV-$0_{B1}$ RNA with RT activity on sucrose gradients was also found with measles and mumps harvests from manufacturer A (data not shown).

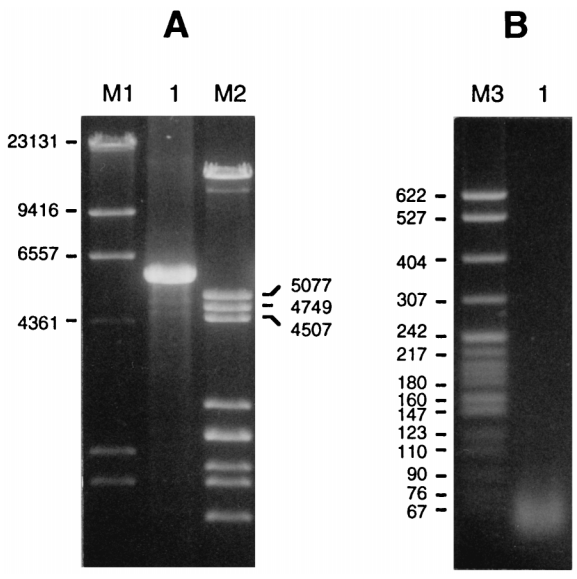


FIG. 6. (A) PCR amplification product of full-length EAV-$0_{B1}$ RNA as described in Materials and Methods. The length is approximately 5.8 kb. M1, size marker λ DNA-*Hin*dIII digest. M2, λ DNA-*Pst*I digest. (B) Demonstration that the cellular U3-R-U5 EAV-0 DNA is absent. DNA was amplified by primers no. 6 and no. 10. No specific product was detectable. M3, pBR322 DNA-*Msp*I digest (several of the bands are identified by length, in base pairs).

**Synthesis of EAV-$0_{B1}$ cDNA by endogenous RT reaction.** The endogenous RT reaction provides the strongest evidence for the presence of retrovirus particles (1, 21). Under the appropriate conditions, the virion's own RT transcribes its own viral RNA within the core. In order to prove the presence of viral particles, which contain both RT and RNA, endogenous cDNA synthesis of an aliquot of gradient fraction no. 10 was measured after rendering the envelopes of particles permeable by low levels of nonionic detergent to allow ingress of dNTPs and oligo$(dT)_{15}$ primers (15, 26). The resulting cDNA product was amplified by 3′ anchored PCR and analyzed by Southern blotting. High levels of endogenous cDNA synthesis occurred at a detergent concentration of 0.01% (Fig. 8, lane 2), whereas low levels of cDNA synthesis were detected at 0% (intact particles) (Fig. 8, lane 1) and 0.4% (disrupted particles) (Fig. 8, lane 3). In the absence of dNTPs during the reverse transcription step, no DNA could be demonstrated (Fig. 8, lane 5), i.e., there was no preexisting DNA and all cDNA was synthesized de novo. The addition of AZT-triphosphate inhibited the activity of the endogenous RT reaction.

**Association of RT with EAV-$0_{B1}$ RNA.** To confirm the close association of RNA and RT, we looked for complexes of tRNA-primed RNA and RT. An aliquot of gradient fraction no. 10 was subjected to high concentrations of nonionic detergent to disrupt the particles. EAV-$0_{B1}$ RNA was hybridized to oligo(dT) beads, and unbound material was washed away. The presumed complexes were incubated with dNTPs to allow cDNA synthesis, and the generated cDNA was amplified by PCR at various regions and analyzed by Southern blotting (Fig. 9). Amplification of the 5′ R-U5 region revealed a strong product (Fig. 9, lane 1), whereas the omission of dNTPs in the reverse transcription step resulted in no amplification product. This demonstrated that the signal generated in lane 1 (Fig. 9) originated from de novo cDNA synthesis. 3′ U3-R-poly(A) amplification also revealed a slight product (Fig. 9, lane 4), but amplification of the *gag*-leader region resulted in no product (Fig. 9, lane 7). This showed that the synthesis of the 5′ R-U5 cDNA didn't start at the $(dT)_{25}$ oligonucleotide by which the EAV-$0_{B1}$ RNA was coupled to the beads but, rather, started upstream of the *gag* sequence. These results strongly suggest

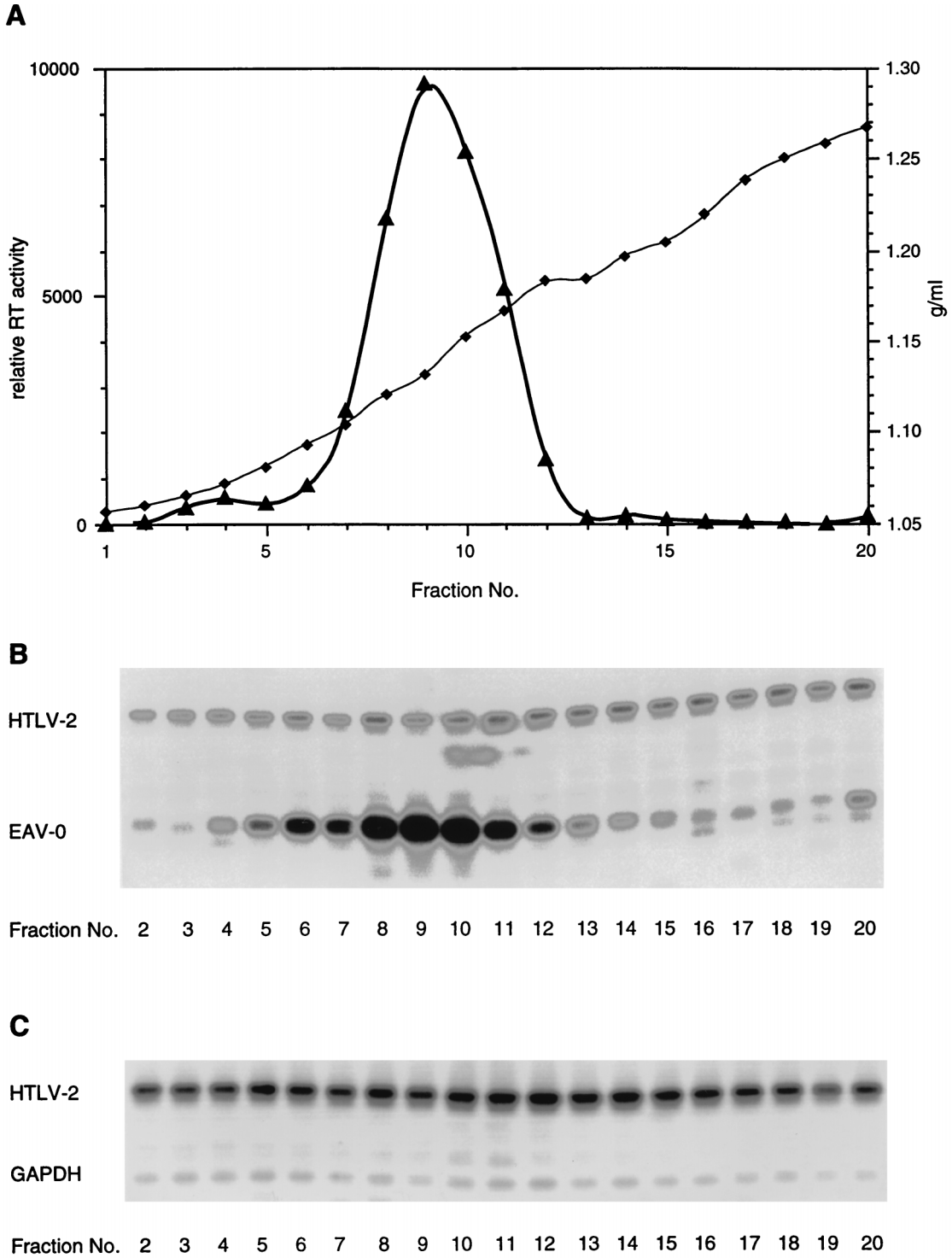


FIG. 7. Colocalization of RT activity and EAV-$0_{B1}$ RNA on sucrose gradient of measles virus harvest (manufacturer B). (A) RT activity (triangles) peaked at a sucrose density (diamonds) of 1.13 g/ml. (B) RNA-PCR of EAV-$0_{B1}$ amplified by 3′ anchored PCR and analyzed by Southern blotting. As an internal control, a constant amount of HTLV-2 was added to an aliquot of each fraction; subsequently, cDNA synthesis and coamplification by 3′ anchored PCR was performed. (C) RNA-PCR of the cellular chicken GAPDH mRNA amplified by 3′ anchored PCR and analyzed by Southern blotting. From each fraction, an identical volume of the cDNA synthesized as described above was subjected to 3′ anchored PCR of GAPDH and HTLV-2 cDNA. The amplification products were visualized on a PhosphorImager.

that the 5′ terminal region of EAV-$0_{B1}$ RNA is naturally primed, probably with host cell-derived $tRNA^{Trp}$, and that it is further associated with RT. Addition of murine leukemia virus RT increased the cDNA synthesis at the 5′ R-U5 region (Fig. 9, lane 3), indicating that not all EAV-$0_{B1}$ RNA was physically associated with endogenous RT or that the complex dissociated during the reaction procedure.

**Quantification of EAV-$0_{B1}$.** Endpoint dilution of peak sucrose gradient fractions no. 6 to 12 of the measles harvest indicated that approximately $4 \times 10^5$ cDNA copies per 1 ml of

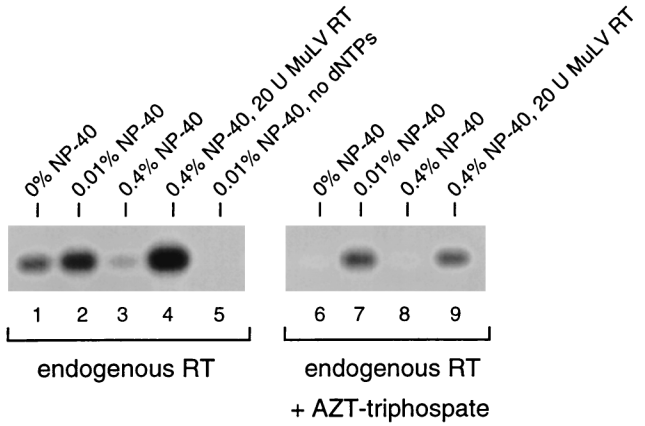


FIG. 8. Synthesis of EAV-$0_{B1}$ cDNA by endogenous RT reaction and inhibition by AZT-triphosphates. A constant volume of gradient fraction no. 10 (Fig. 7) was incubated with buffer, $dT_{15}$ primer, dNTPs, and various detergent concentrations. The generated cDNA was amplified by 3′ anchored PCR and analyzed by Southern blotting. The amplification products were visualized on a PhosphorImager. Endogenous RT activity was inhibited by the addition of the RT-specific inhibitor AZT-triphosphate. MuLV, murine leukemia virus (SuperScript II RNaseH$^-$ RTase [Gibco BRL]).

virus harvest were present. Assuming a 50% cDNA synthesis efficiency, the $4 \times 10^5$ cDNA molecules correspond to $8 \times 10^5$ RNA molecules or $4 \times 10^5$ virus particles, under the assumption that the particles contain two copies of RNA.

## DISCUSSION

We recently reported the detection of RT activity in chicken cell-derived live attenuated virus vaccines (7); this finding was quickly confirmed by several other groups (25). Although the members of the World Health Organization Expert Committee on Biological Standardization agreed with the opinion that this enzyme activity in vaccines presents no harm to vaccinees or to the population at large, there was also agreement that a rapid identification and biological characterization of the agent(s) responsible for the RT activity was necessary (17, 25).

In theory, several sources of this RT activity are conceivable. It may be associated with endogenous or exogenous retroviruses, cellular enzymes like DNA or RNA polymerases or telomerases, or cellular elements like retrotransposons. For several reasons, we favored a retroviral origin. The activity could be sedimented and filtered, it peaked at a density consistent with that of retroviruses, and it was inhibited by both AZT-triphosphate and ddTTP at inhibitor concentrations that did not affect the activity of *Taq* DNA polymerase. The enzyme exhibited a preference for $Mg^{2+}$, which is the preference of avian retrovirus RT. Since the activity was present in all vaccines produced in cells of chicken origin, independent of manufacturer, lot, or production year, we favored an endogenous rather than exogenous virus (for an extended discussion, see reference 7).

In our approach to identify the origin of this RT activity, we made use of a novel method that permits the selective and efficient amplification of particle-associated RNAs of a wide range of retroviruses present at low concentrations (22). The use of primers derived from $tRNA^{Pro}$, $tRNA_3^{Lys}$, $tRNA_{1,2}^{Lys}$, or $tRNA^{Trp}$ in this procedure permits amplification and identification of all exogenous retroviruses identified to date, as well as endogenous retroviruses, which are expressed and possess one of the above-noted tRNA PBs, without a need to perform even a single virus-specific test. Since all avian retroviruses identified to date, including the endogenous ones, posses a tryptophan PB, the finding that amplifications with primers complementary to the tryptophan PB repeatedly yielded a product, while amplification with other primers did not, was not unexpected (Fig. 4). A 5′ R-U5 sequence of the EAV-0 family, termed EAV-$0_{B1}$, was repeatedly isolated from the RT peak fraction of a sucrose gradient-purified measles virus harvest, while sequences of other avian retroviruses were not found. Thus, there was no indication that the RT activity of this vaccine was associated with a virus of the avian leukemia-sarcoma virus (ALSV) group. A theoretical possibility that a virus other than EAV-0 might also be associated with this activity, namely, one which uses a tRNA PB different from the four we tested here, cannot at present be completely ruled out.

The presence of viral genomic EAV-$0_{B1}$ RNA was demonstrated based on the redundancy of the R region at both ends of the sequence, the 3′ polyadenylation (Fig. 5), and an estimated length of 5.8 kb, which is comparable to the length of the proviral EAV-0 DNA of about 5.8 kb (19). This RNA was specifically enriched in the sucrose gradient fractions which contained peak RT activity (Fig. 7), it was enclosed together with RT in the same membranated compartments (Fig. 8), and it was naturally primed and physically associated with RT (Fig. 9). Taken together, these results provide strong evidence that CEF-derived virus harvests, as well as the control harvests which exhibit similar levels of RT activity (Fig. 2), contain retrovirus-like particles that harbor both EAV-0 RNA and an enzymatically active RT. Since there was no evidence for any other retrovirus RNA, these findings are best compatible with the hypothesis that an EAV-0 provirus may be capable of producing RT-positive particles.

EAV-0 proviral sequences were first isolated from the genomic DNA of *ev*$^-$ line 0 chickens, which lack endogenous avian leukosis virus loci (12). EAV-0 sequences are present at approximately 50 copies per haploid genome of the avian genus *Gallus* (13) and were shown to be transcriptionally active during embryogenesis of line 0 chickens (9). In view of the experimental evidence now suggesting that EAV-0 may even be capable of producing RT-positive particles, the question arises whether the never-identified, particle-associated RT ac-

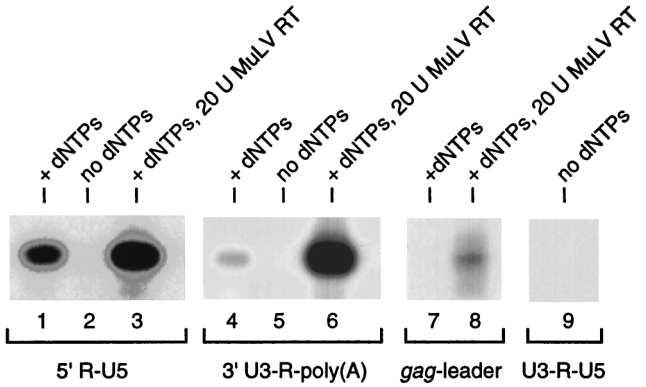


FIG. 9. RT is physically associated with EAV-$0_{B1}$ RNA. Triplicates of a volume of the sucrose fraction no. 10 were treated with 0.5% detergent, and viral RNA was hybridized to oligo(dT) magnetic beads. The RNA bound to the beads was washed and incubated in three different ways, i.e., with buffer containing dNTPs, no dNTPs, or dNTPs with the addition of 20 U of murine leukemia virus (MuLV) RT. The generated cDNA was amplified by PCR of the 5′ R-U5 region, anchored PCR of the 3′ U3-R-poly(A) region, PCR of the *gag*-leader region, or PCR of the U3-R-U5 region, as described in Materials and Methods, and analyzed by Southern blotting. The amplification products were visualized on a PhosphorImager.

tivity found 20 years ago by Bauer et al. in the allantoic fluid of embryonated chicken eggs (3) and in the supernatant of primary chick embryo cell cultures derived from ALSV-free chickens (5) is caused by the presence of EAV-0 particles. This is probable, because Bauer et al. observed that their purified RT-positive particles exhibited a preference for $Mg^{2+}$ (3) and banded at a lower density than the retroviruses of the ALSV group (4), which is in agreement with our results (Fig. 7) (7). Bauer et al. further reported that their particles sedimented more slowly and lacked infectivity (4). These properties are consistent with the fact that several independently isolated EAV-0 clones each showed a unique deletion of approximately 1.2 kb in *env*, which spanned a region extending from around the signal peptide of the surface protein to the middle of the coding region of the transmembrane protein (8). In addition, the particle-associated RT activity released by chick embryo cell cultures depended on the age of the embryo from which the cultures were established and on the time during which the cells had been in culture (5). These observations are compatible with the transcriptional activity of EAV-0 during embryogenesis of line 0 chickens (9) and provide a possible explanation for the extensive variation in RT activity we found between different harvests of the same manufacturer (Fig. 2).

The fact that Bauer et al. were unable to demonstrate an association of their particles with RNA or endogenous RT activity (5) seems at odds with our findings (Fig. 7 and 8). They reasoned that their negative results could be due to the limited amount of material, the experimental conditions applied, or the destruction of the template during purification of the particles. The enormous increase in sensitivity achieved meanwhile by PCR-based methods could explain the discrepancy. Additional possibilities are that not all chicken lines harbor EAV-0 proviruses that are capable of producing particles which contain RNA or that only a fraction of these particles contain RNA.

Visualization of particles with a typical morphology by electron microscopy would provide further strong support for the existence of retrovirus particles of EAV-0. However, since the quantification of EAV-$0_{B1}$ RNA indicated the presence of only about $4 \times 10^5$ virus particles/ml, it will be almost impossible to prove their existence by this technique. Thus, final proof will have to come from molecular techniques.

Similarly, although our demonstration that RT and EAV-$0_{B1}$ RNA are present in the same compartment and are physically associated strongly suggests that EAV-$0_{B1}$ encodes this RT, these findings are not formal proof of this. Attempts to clone and express the EAV-$0_{B1}$ RT are in progress in order to compare the recombinant RT with the activity found in the live attenuated harvests.

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