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Molecular Cloning and Characterization of *gag*-, *pol*-, and *env*-Related Gene Sequences in the *ev*$^-$ Chicken

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Using less stringent hybridization conditions and cloned viral DNA probes representing the avian sarcoma virus *gag*, *pol*, *env*, and long terminal repeat (LTR) gene sequences, we detected related sequences in two avian species purportedly lacking all endogenous avian leukosis viruses, the *ev*$^-$ chicken and the Japanese quail. The blot hybridization patterns obtained with the various probes suggest the presence of between 40 and 100 copies of retrovirus-related sequences in the genomes of these two species. An *ev*$^-$ chicken genomic DNA library was prepared and screened with *gag*-specific and *pol*-specific DNA probes. Several different clones were obtained from this library and characterized. Analysis of these clones revealed that the retrovirus-related gene sequences are linked in the order LTR-*gag*-*pol*-*env*-LTR, a structure indicative of a complete provirus. These data indicate the presence of previously unidentified endogenous retrovirus species in avian cells, suggesting that under the appropriate conditions of hybridization additional, more distantly evolved families of endogenous retrovirus genes may be identified in vertebrate species.

The genomes of endogenous retroviruses are found in multiple copies in the chromosomes of most vertebrate species (4, 18, 27, 28). These genes are present in all cells of all tissues of an animal and are inherited in a simple Mendelian fashion. The chicken genome carries at least 16 endogenous Rous-associated virus-0-related proviral copies (*ev-1* through *ev-16*), each located at a unique chromosomal site (3, 23). Some endogenous proviral loci are able to specify the synthesis of infectious virus (6), whereas others represent deleted genomes capable of coding for only a subset of retrovirus proteins (1, 5). In the extreme case, the only viral sequences remaining consist of a single long terminal repeat (LTR).

Endogenous viral genes have been shown to be essentially ubiquitous in all vertebrate species, including humans. This observation, as well as the association of their expression with the development of the hematopoietic system in mice (14, 26), has led to the proposal that these genes are involved in ontogeny. However, the reported successful breeding of a healthy, fertile chicken lacking all avian leukosis virus (ALV)-related endogenous viral genes (*ev*$^-$) is considered strong evidence against such a hypothesis (2). It has also been suggested that endogenous viruses may play a more evolutionary role, conferring a selective advantage on a host animal exposed to infectious virus particles (7, 22), as evidenced by the unusual susceptibility of chickens free of endogenous viruses (*ev*$^-$) to infection with exogenous retroviruses. In this report, we describe the cloning and characterization of a new family of endogenous viral genes present in both the *ev*$^-$ chicken and the Japanese quail, indicating that the *ev*$^-$ chicken does, in fact, contain a more distantly evolved family of endogenous proviruses.

MATERIALS AND METHODS

Cloned viral DNAs. *gag*- and *env*-specific probes were generated by subcloning gene-specific restriction fragments from the avian sarcoma virus (ASV) clone pATV8 (13). The *gag*-specific probe was constructed by subcloning the 1.3-kilobase (kb) *Bam*HI-*Bam*HI restriction fragment into the vector pUC-9, and the *env*-specific probe was constructed by subcloning a 1.7-kb *Xho*I-*Xho*I restriction fragment into the vector pUC-17. The plasmids were designated pUC-*Gag* and pUC-*Env*, respectively. The *pol*-specific probes used were pUC-RT and pUC-*ev*$^-$-RT (10). The LTR-specific probe was generated by subcloning a 340-base-pair (bp) *Eco*RI-*Eco*RI restriction fragment from the ASV clone λRPA102 (11) into the vector pUC-9; it was designated pUC-LTR.

Preparation of DNA probes. All Southern blot data were generated by using gel-purified DNA fragments as probes. The various plasmids were digested with the restriction endonucleases needed to liberate the inserts. The probe-specific fragments were then isolated from all other plasmid sequences by electroelution onto DE-81 paper in agarose gels. The eluted DNA was then resuspended in water and labeled by nick translation (20) to specific activities of 2×10^8 to 4×10^8 cpm/μg.

Preparation of cellular DNAs. High-molecular-weight genomic DNA was extracted from 11-day-old White Leghorn chicken embryos (*ev*$^+$ and *ev*$^-$), 8-day-old Japanese quail embryos, and ASV-transformed chicken embryo fibroblasts as described previously (10). *Drosophila melanogaster* DNA was kindly provided by M. Blumenfeld, University of Minnesota.

Construction of *ev*$^-$ chicken genomic library. High-molecular-weight DNA extracted from *ev*$^-$ chicken embryos was digested under partial digestion conditions with *Eco*RI and fractionated on a 0.7% agarose gel. DNA fragments ranging in size from 12 to 18 kb were eluted onto DE-81 paper and purified. A sample of this DNA was then ligated with *Eco*RI-digested, gradient-purified arms of the vector λL47.1 (15). The ligation mixture was directly packaged into bacteriophage coats (17) and plated on the selective bacterial host Q359. Nitrocellulose lifts were made from the plates and hybridized either stringently with the probe pUC-*ev*$^-$-

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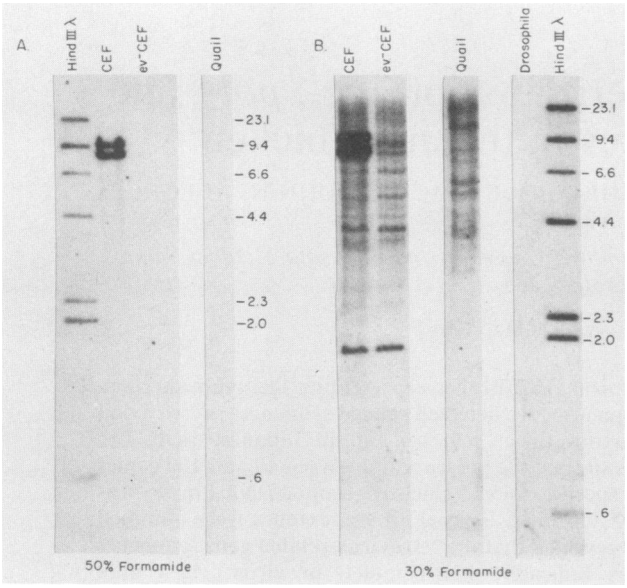

FIG. 1. *gag*-related sequences in chicken and quail DNA. DNAs extracted from ev^+ chicken embryos, ev^- chicken embryos, and quail embryos were digested with *Eco*RI, and 10 μg of DNA per lane was fractionated by agarose gel electrophoresis. The DNAs were transferred to nitrocellulose filters and hybridized to a nick-translated *gag*-specific probe under stringent (50% formamide) (A) and less stringent (30% formamide) (B) hybridization conditions. *Eco*RI-digested *Drosophila* DNA (5 μg) was included in panel B as a negative control. Size markers are in kilobases and represent *Hin*dIII-digested phage λ DNA. CEF, Chicken embryo fibroblast.

RT or less stringently with the probe pUC-*Gag*. Plaques showing hybridization were picked and plaque purified three times. Plate lysates were used to prepare DNA for analysis (16). In addition, a more complete ev^- chicken genomic library was constructed by using the lambda vector Charon 30 (21). *Mbo*I was used to digest ev^- chicken genomic DNA under partial digestion conditions, and DNA fragments ranging in size from 15 to 20 kb were purified from sucrose gradients (17). Charon 30 DNA was digested to completion with *Bam*HI and *Sal*I, and the arms were purified from the internal stuffer fragments by sucrose gradient fractionation. A sample of the ev^- chicken DNA was then ligated with the purified arms of Charon 30, and the resulting molecules were packaged in vitro and plated out on the *Escherichia coli* host K802 for screening.

Cloning of 5.2-kb single-copy clone. High-molecular-weight ev^- chicken genomic DNA (50 μg) was digested to completion with *Eco*RI and fractionated by agarose gel electrophoresis. Fragments ranging in size from 4.3 to 7.0 kb were selectively eluted onto DE-81 paper. The purified ev^- chicken DNA was ligated with *Eco*RI-digested arms of the lambda insertion vector Charon 16A (29), packaged in vitro, and plated out on *E. coli* LE392 for screening. The recombinant phage population was screened with the eluted 1.3-kb *pol* probe derived from the plasmid pUC-ev^--RT under stringent hybridization conditions.

Nucleic acid hybridization. DNAs were digested with restriction endonucleases, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose filter paper by a modification of the Southern procedure (25). Hybridizations and washes were done under stringent conditions (50% formamide) or less stringent conditions (30% formamide), as described previously (10), except that dextran sulfate was not used.

DNA sequence analysis. DNA fragments for sequencing were generated by a combination of two procedures. First, specific restriction fragments were cloned directly into the M13 vectors mp8 and mp9. Second, deletion variants of the original clones were generated by the procedure of Dale et al. (8). Single-stranded template DNA was prepared and subjected to DNA sequence analysis by using the chain-termination procedure of Sanger et al. (24). DNA was labeled with [^{35}S]dATP by the primer extension reaction in the presence of dideoxynucleoside triphosphate analogs. The resulting products were analyzed on 6 and 8% polyacrylamide gels. DNA sequence comparisons were done with the National Institutes of Health program NUCALN.

RESULTS

***gag*-, *pol*-, and *env*-related sequences in avian DNA.** Using a cloned viral DNA probe specific for the ASV *gag* gene, we screened DNA from two avian species for the presence of *gag*-related nucleotide sequences. The probe was hybridized to filters containing *Eco*RI-digested DNAs extracted from ev^+ chicken embryos, ev^- chicken embryos, and Japanese quail embryos under both stringent and less stringent hybridization conditions. *Eco*RI-digested DNA from *D. melanogaster* was included as a negative control for nonspecific hybridization under the less stringent conditions. Under the stringent hybridization conditions (Fig. 1A), the only bands detected were the *gag*-specific bands expected from the endogenous ALVs in the ev^+ chicken embryos. Neither the DNA from the ev^- chicken embryos nor the DNA from the Japanese quail embryos revealed any detectable sequence homology under these conditions; these results are consist-

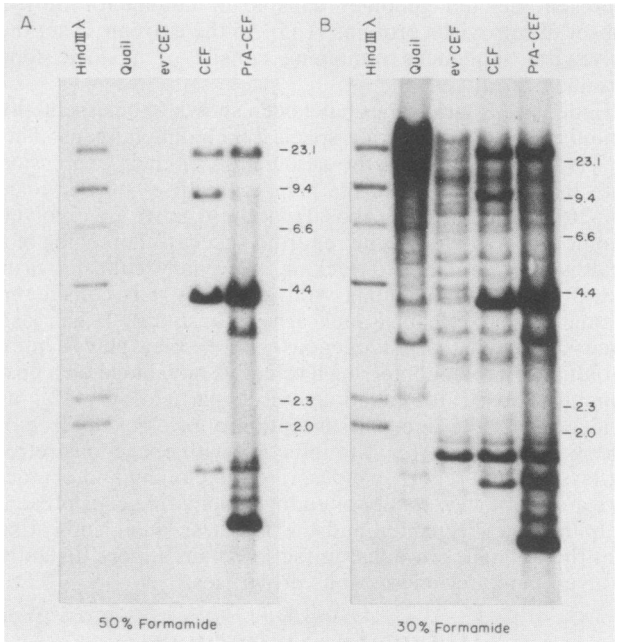

FIG. 2. *env*-related sequences in chicken and quail DNA. Hybridizations were done as described in the legend to Fig. 1 with two exceptions: DNA extracted from Prague A (PrA) ASV-infected chicken embryo fibroblasts (CEFs) was included and the probe used represented *env*-specific sequences. Size markers are in kilobases and represent *Hin*dIII-digested phage λ DNA.

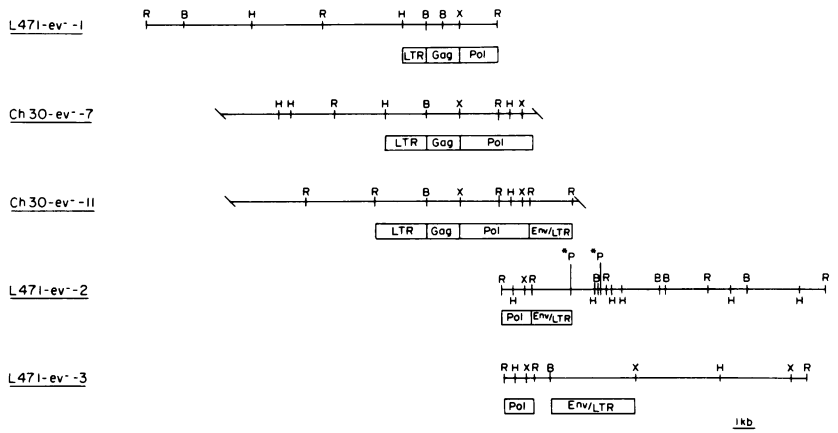

FIG. 3. Genetic structure of ev^- chicken genomic clones. Five distinct genomic clones were mapped by restriction enzyme analysis and Southern blotting. The regions of the clones that hybridized with the different viral probes are indicated by bars below the restriction maps. The diagonal lines at the ends of the Charon 30 clones represent the estimated junctions between the lambda-derived arms and the inserts. The restriction enzymes used were as follows: R, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; X, *Xho*I; P, *Pst*I. Asterisks denote those sites mapped within the 3.2-kb *Eco*RI-*Eco*RI fragment of clone L47.1-ev^--2 only.

ent with previous findings indicating the lack of endogenous ALVs in these two species (2).

When less stringent hybridization conditions were used (Fig. 1B), both the ev^- and ev^+ chicken embryos revealed an identical pattern of positively hybridizing restriction fragments that were not detected previously. The Japanese quail embryo DNA also revealed a new array of *gag*-related restriction fragments that were not detected under stringent hybridization conditions. No hybridization could be detected between the *gag*-specific probe and the *Drosophila* DNA, indicating that the fragment pattern detected under less stringent hybridization conditions was not due to fortuitous cross-annealing between the probe and cellular sequences.

When the same DNAs, as well as the DNA extracted from Prague A ASV-infected chicken embryo fibroblasts, were screened with a probe specific for the ASV *env* gene, similar results were obtained. The only bands detected under stringent hybridization conditions (Fig. 2A) were those representing either endogenous proviral sequences or the exogenous proviral sequences introduced through infection. However, when the hybridizations were performed under less stringent conditions, a new and identical array of *env*-specific restriction fragments was detected in all chicken DNA samples (Fig. 2B). *env*-related sequences were also detected in Japanese quail under these conditions. These data taken collectively with our previously published data generated by the use of a *pol*-specific probe (10) suggest that we have identified either distantly related, cellular homologs to the avian retroviral genes or a more distantly related family of endogenous proviruses. In an effort to distinguish between these two possibilities, we attempted to determine whether the *gag*-, *env*-, and *pol*-related sequences detected are linked together in a structure indicative of a provirus.

Cloning and characterization of retrovirus-related sequences in the ev^- chicken. A genomic library was constructed by using the lambda substitution vector λL47.1 (15) and purified DNA from ev^- chicken embryos. Partially *Eco*RI-digested ev^- chicken embryo DNA was fractionated on an agarose gel, and fragments ranging in size from 12 to 18 kb were electroeluted from the gel. The resulting fragments were then ligated with gradient-purified arms of λL47.1 and packaged in vitro, and recombinants were selected by using the phage P2 lysogenic host Q359. The library was screened under relaxed hybridization conditions with the ASV *gag*-specific probe. In addition, a 1.3-kb *Eco*RI fragment representing the major *pol*-related band detected in the DNA blots of ev^- chicken embryos which had previously been cloned (10) was used to screen the library under stringent hybridization conditions. Three separate clones (designated L47.1-ev^-) were isolated and mapped by restriction enzyme analysis and Southern blotting (Fig. 3). One clone, ev^--1, was shown to have a 740-bp *Bam*HI-*Bam*HI fragment and a 630-bp *Bam*HI-*Xho*I fragment which hybridized to a *gag*-specific probe (Fig. 4A). In addition, a 1.55-kb *Xho*I-*Eco*RI fragment, located adjacent to the *gag*-related region, hybridized to the *pol*-specific probe, indicating a *gag-pol* linkage (Fig. 4B). Two other selected clones, ev^--2 and ev^--3, which failed to hybridize with the *gag*-specific probe, hybridized to both the ASV *pol*- and *env*-specific probes. Both clones contain a 1.3-kb *Eco*RI-*Eco*RI restriction fragment that hybridized to the *pol* probe. *env*-related sequences could be localized to a 1.7-kb *Eco*RI-*Pst*I fragment and a 3.7-kb *Bam*HI-*Xho*I fragment (Fig. 3) within clones ev^--2 and ev^--3, respectively. These data reveal that both clones contain *pol*-specific sequences adjacent to *env*-specific sequences, indicating a *pol-env* linkage.

The data for the three clones described above suggest the presence of complete retroviral provirus structures in the DNA of ev^- chickens. However, the approach used to generate these clones may have selected against a complete proviral copy because of the nonrandom location of *Eco*RI restriction sites throughout the genome. To more completely characterize the ASV-related sequences in the ev^- chicken genome, we constructed a second genomic library calculated to contain clones representing the entire ev^- chicken genome. Briefly, ev^- chicken genomic DNA was digested with *Mbo*I under partial digestion conditions to generate an overlapping and random class of restriction fragments. Fragments ranging in size from 15 to 20 kb were purified from sucrose gradients (17) and ligated with gradient-purified arms of *Bam*HI-digested Charon 30 DNA (21). The resulting molecules were packaged in vitro and plated on the host K802 for screening. The library was specifically screened before amplification to ensure that those components which had any growth advantage were not overrepresented. The resulting library was screened under stringent hybridization conditions with the 1.3-kb *pol*-specific fragment from the

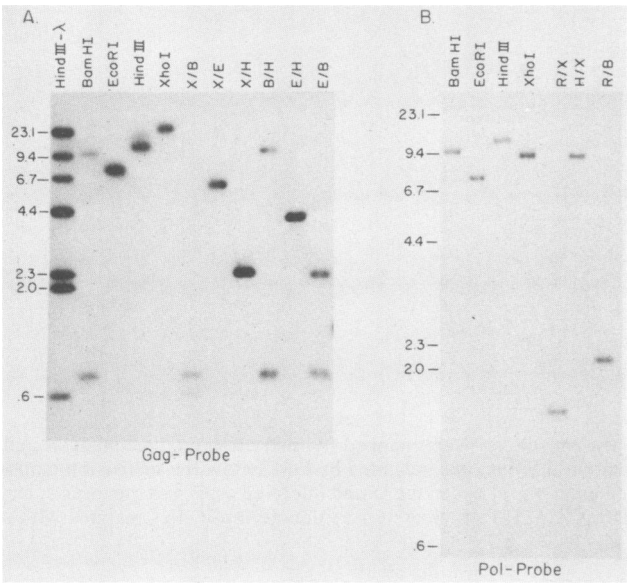

FIG. 4. Southern blot analysis of clone L47.1-ev^--1. Equal amounts of L47.1-ev^--1 DNA (0.5 μg per lane) were treated with various restriction endonucleases in single and double digests, fractionated by electrophoresis, and transferred to nitrocellulose. The filter was hybridized with the *gag*-specific portion of pUC-*Gag* (A) or the *pol*-specific portion of pUC-RT (B) under relaxed hybridization conditions. The size markers represent ^{32}P-labeled *Hin*dIII-digested phage λ DNA and are in kilobases. X, *Xho*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII.

plasmid pUC-ev^--RT. Two clones were isolated and characterized by restriction enzyme analysis and Southern blotting (Fig. 3). One clone, Ch30-ev^--7, was shown to contain *gag*-related sequences linked to *pol*-related sequences. The *pol*-related sequences identified in this clone spanned the junction between the *pol*-related sequences identified in clones L47.1-ev^--1, L47.1-ev^--2, and L47.1-ev^--3, suggesting that those sequences may be joined at their *Eco*RI sites. A second distinct clone, Ch30-ev^--11, appeared to contain a complete proviral structure with *gag*-, *pol*-, and *env*-related sequences linked to one another in the order expected for an endogenous provirus.

Since these data suggested a linkage arrangement reminiscent of a complete proviral structure, we decided to determine if LTR-like sequences could be identified upstream of the *gag*-related sequences and downstream of the *env*-related sequences. This analysis revealed that clones L47.1-ev^--1, Ch30-ev^--7, and Ch30-ev^--11 contain LTR-related sequences upstream of and directly adjacent to the *gag*-related sequences (Fig. 3). Furthermore, clones L47.1-ev^--2, L47.1-ev^--3, and Ch30-ev^--11 all contain LTR-related sequences in the restriction fragment containing the *env*-related sequences. Because of the lack of restriction sites within this region, more precise mapping of the LTR-related sequences relative to the *env*-related sequences could not be performed. However, these fragments are large enough to code for both envelope and LTR sequences. The presence of LTR-related sequences flanking both the *gag*- and *env*-related sequences on clone Ch30-ev^--11 further suggests that this clone may represent a complete proviral structure.

Identification and cloning of a single-copy *pol*-related fragment from the ev^- chicken. The complex pattern of hybridization obtained when chicken genomic DNAs were hybridized with ASV-derived probes suggested that these ASV-related sequences might be present at rather abundant copy numbers. To resolve this issue, we attempted to determine the relative copy number of the *pol*-related sequences present in the chicken DNA and also to determine if any new *pol*-related sequences could be detected with the plasmid pUC-ev^--RT that were not detected with the ASV-derived *pol* probe (pUC-RT). Various amounts of the ev^- chicken DNA were digested with either *Eco*RI or *Bam*HI and then electrophoresed in parallel with increasing amounts of *Eco*RI-digested pUC-ev^--RT calculated to represent between 4 and 100 copies per cell. The resulting DNAs were blotted to nitrocellulose and hybridized with the gel-purified, nick-translated 1.3-kb *pol*-specific insert derived from the plasmid pUC-ev^--RT.

Hybridizations were performed under both stringent and less stringent conditions. The results (Fig. 5) demonstrate that the hybridization pattern obtained with the *Bam*HI-digested DNA under stringent conditions was identical to the pattern obtained with the ASV *pol*-derived probe under less stringent conditions (results not shown). This demonstrates that all the *pol*-related fragments detected with the ASV-derived *pol* probe represented authentic *pol*-related sequences and were not results of fortuitous cross-hybridization between the probe and cellular sequences under the less stringent hybridization conditions. Furthermore, the blot hybridization pattern obtained with the 1.3-kb ev^--derived *pol* probe under less stringent hybridization conditions in an attempt to identify an even more distantly

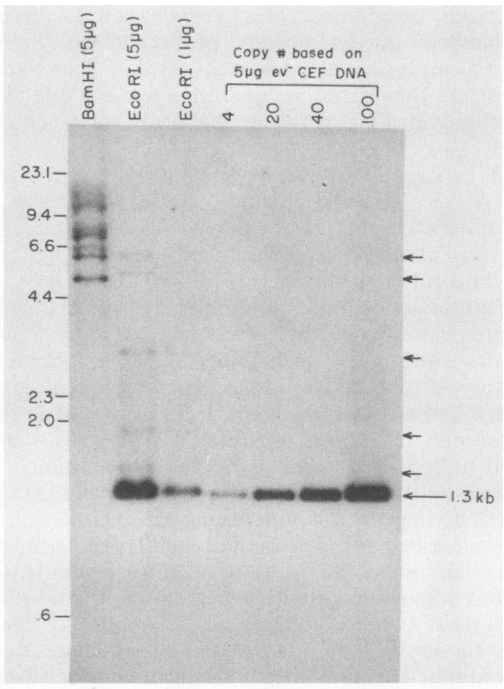

FIG. 5. Copy number estimates for *pol*-related sequences in the ev^- chicken. Various amounts of ev^- chicken DNA were digested with either *Eco*RI or *Bam*HI, fractionated on an agarose gel, and blotted to nitrocellulose. Increasing amounts of *Eco*RI-digested pUC-ev^--RT were run in parallel as copy number controls, and the blot was hybridized to the 1.3-kb ev^- *pol*-related fragment under stringent hybridization conditions. The major 1.3-kb *pol*-related fragment is marked by a large arrow, and the five single-copy fragments are marked by small arrows. Size markers are in kilobases and represent *Hin*dIII-digested phage λ DNA. CEF, Chicken embryo fibroblast.

related family of endogenous viruses revealed no new bands (data not shown). This suggests that the *pol*-related sequences detected with the ASV-derived *pol* probe represent most, if not all, of the retroviral *pol*-related sequences in the ev^- chicken. When the *Eco*RI and *Bam*HI digests were compared with the copy number standards, a copy number of between 40 and 100 copies per cell was estimated. This indicates that the *pol*-related sequences identified in the ev^- chicken represent a family of moderately repetitive sequences. In addition to the 1.3-kb fragment identified in the *Eco*RI digest, five higher-molecular-weight bands were detected (Fig. 5, arrows) at much lower copy numbers. These fragments appear to be present at between one and two copies per cell. Because these fragments were characteristic of what is expected for a unique-sequence cellular gene and because they were different in size from the 1.3-kb fragment found to be part of an endogenous viral structure, an attempt was made to clone and characterize one of these single-copy *pol*-related fragments to determine whether it might represent cellular sequences related to the retrovirus reverse transcriptase gene.

High-molecular-weight ev^- chicken genomic DNA (50 μg) was digested to completion with *Eco*RI and fractionated by agarose gel electrophoresis. Fragments ranging in size from 4.3 to 7.0 kb were eluted onto DE-81 paper. The purified ev^- chicken DNA was ligated with *Eco*RI-digested arms of the lambda insertion vector Charon 16A (29), packaged in vitro, and plated out on the *E. coli* host LE392. Approximately 10^5 plaques were screened with the eluted 1.3-kb *pol* probe derived from the plasmid pUC-ev^--RT, and a single positive clone was identified. This clone (designated 16A-ev^--5.2) was plaque purified and then mapped by restriction enzyme analysis and Southern blotting. The restriction enzyme map of the 5.2-kb *pol*-related insert from clone 16A-ev^--5.2 and the regions of the clone that hybridized with the various ASV-derived probes are shown in Fig. 6. *pol*- and *env*-related sequences were detected adjacent to one another and localized to a 750-bp *Bgl*II-*Pst*I fragment (Fig. 6A) and a 1.7-kb *Pst*I-*Hin*dIII fragment (Fig. 6B), respectively. In addition, the LTR-specific probe also hybridized to the 1.7-kb *Pst*I-*Hin*dIII fragment, indicating the presence of an LTR flanking the *env*-related sequences (data not shown). No *gag*-related sequences were detected on the 5.2-kb fragment. When clone 16A-ev^--5.2 was mapped by using the 1.3-kb *pol*-related fragment from pUC-ev^--RT as the probe, homology was localized to the 1.3-kb *Eco*RI-*Pst*I fragment (data not shown). This region shared a *Bgl*II site with the 1.3-kb major *pol*-related fragment but appeared to be missing sites for *Hin*dIII, *Xho*I, and *Eco*RI relative to this fragment. These data indicate that the 5.2-kb single-copy, *pol*-related clone represents a portion of a different provirus which differs from the major *pol*-related fragment because of restriction enzyme polymorphisms.

Partial nucleotide sequence analysis of the *gag*- and *env*-related sequences cloned from the ev^- chicken. We previously demonstrated that the *pol*-related sequences detected by our low-stringency hybridization conditions reflect significant homologies at both the DNA and amino acid sequence levels (10). To extend these results to the *gag*- and *env*-related sequences discussed in this study, portions of the *gag*-specific region from clone L47.1-ev^--1 and the *env*-specific region of clone L47.1-ev^--2 were subjected to nucleotide sequence analysis by the procedure of Sanger et al. (24). The resulting sequences were then compared to the complete nucleotide sequence of Rous sarcoma virus (RSV). Region I, which is located within the 740-bp *Bam*HI-*Bam*HI fragment

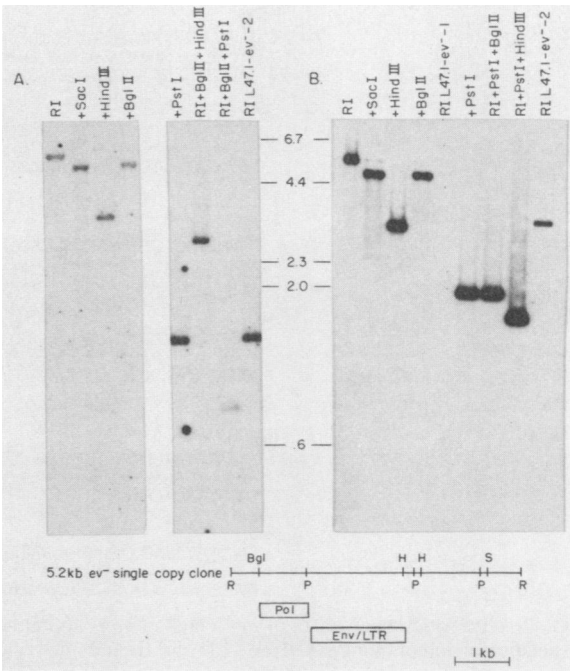

FIG. 6. Characterization of single-copy *pol*-related clone Charon 16A-ev^--5.2. The 5.2-kb single-copy, *pol*-related fragment cloned from an ev^- chicken was mapped with a variety of restriction enzymes. The portions of the cloned fragments homologous with viral *pol* (A) and *env* (B) probes were determined by Southern blot analysis. The restriction map of the 5.2-kb fragment is shown, and those regions of the fragment homologous with the various probes are indicated by bars. Lanes marked with a plus followed by a single enzyme were digested in combination with *Eco*RI. L47.1-ev^--2 was included as a positive control for both *pol* and *env* sequences. L47.1-ev^--1 was included as a negative control. Size markers are in kilobases and represent *Hin*dIII-digested phage λ DNA. R, RI, *Eco*RI; P, *Pst*I; H, *Hin*dIII; S, *Sac*I.

of L47.1-ev^--1 (Fig. 3), reveals 63% DNA sequence homology with the P19 portion of the RSV *gag* gene (Fig. 7). Region II is located in the middle of the 1.55-kb *Eco*RI-*Xho*I fragment from clone L47.1-ev^--1 (Fig. 3). This region has 60% homology with a portion of the RSV genome spanning the *gag-pol* junction. Region III is derived from the middle of the *Eco*RI-*Pst*I fragment of clone L47.1-ev^--2 that revealed hybridization with the viral *env*-specific probe. When this sequence was compared with the entire RSV sequence, it exhibited 62% DNA sequence homology with the gp37 portion of the *env* gene. These results demonstrate that the homologies detected between the cellular sequences and the viral probes used in this study represent true and significant homologies at the nucleotide sequence level.

DISCUSSION

Endogenous viruses have been identified in every vertebrate species analyzed for their presence, including humans. Their apparent ubiquity, as well as the reported association of their expression with the development of the hematopoietic system in mice, has led to the hypothesis that these genes play some role in the normal development of organisms. However, the reported breeding of a healthy, fertile, normally developed chicken, purportedly lacking all endogenous ALVs (2), strongly suggested that they are not required for normal development. This led to further proposals

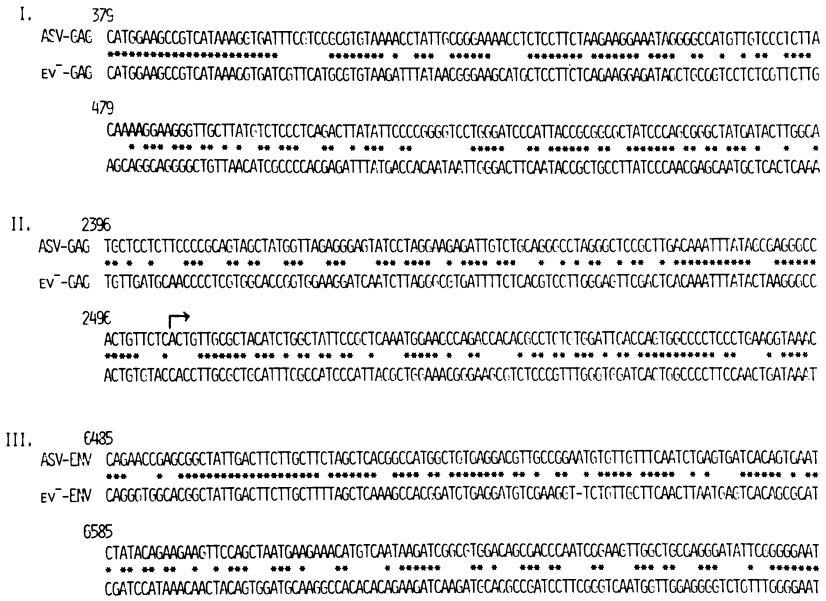

FIG. 7. Nucleotide sequences of *ev*⁻ chicken *gag*- and *env*-related sequences and Prague C ASV genome. Two distinct regions of the *gag*-specific portion of clone L47.1-*ev*⁻-1 (I and II) and one region of the *env*-specific portion of clone L47.1-*ev*⁻-2 (III) were sequenced. The sequences were then compared with the complete nucleotide sequence of the Prague C ASV genome and aligned to give the best fit by using the NUCALN program. The upper line of each row represents the Prague C ASV sequence, and the lower line represents the sequences derived from the chicken clones. The numbering corresponds to the Prague C ASV sequence, with position 1 being the first base of the genomic RNA. Identical bases are indicated by an asterisk. The arrow indicates the start of the *pol* gene relative to the RSV sequence.

suggesting that endogenous viruses played an evolutionary role, providing a selective advantage to the organisms carrying them. Lastly, it has been suggested that endogenous proviruses represent parasitic pieces of DNA (9, 12, 19).

The data which we presented here refute the claim that the *ev*⁻ chicken lacks all endogenous viruses. We identified *gag*-, *pol*-, *env*-, and LTR-related sequences previously undetected in *ev*⁻ chickens and showed that they are linked on a single contiguous piece of DNA in the order expected for a proviral structure. We demonstrated that the *gag*- and *env*-related sequences detected by this type of analysis are significantly homologous at the DNA sequence level, as we previously demonstrated for the *pol*-related sequences (10). This novel family of endogenous viruses appears to represent a moderately repetitive family of DNA elements, as demonstrated by the presence of between 50 and 100 copies per cell of the major 1.3-kb *pol*-related fragment.

The role, if any, played by endogenous proviruses during the development or evolution of vertebrate species is unclear. However, the identification in this report of a more distantly related family of endogenous ALVs present in the *ev*⁻ chicken, as well as in the Japanese quail, requires the reassessment of their potential importance.

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