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Phylogenetic Distribution of the Novel Avian Endogenous Provirus Family EAV-0

ROBERT M. RESNICK,[1]† MICHAEL T. BOYCE-JACINO,[2] QIN FU,[2,3] AND ANTHONY J. FARAS[1,2]*

Institute of Human Genetics,[2] Department of Microbiology,[1] and the Department of Genetics and Cell Biology,[3] University of Minnesota, Minneapolis, Minnesota 55455

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A new family of related endogenous proviruses, existing at 50 to 100 copies per haploid genome and distinguishable by remarkably short long terminal repeats, has been described for domestic chickens (*Gallus gallus* subsp *domesticus*). In this communication, by using Southern blot analysis and probes derived from both internal viral sequences and locus-specific, cellular flanking sequences, we studied the genetic distribution of this family of moderately repetitive avian endogenous retroviruses within the genomes of four *Gallus* species. Eight inbred lines of domestic chickens, the evolutionary progenitor to the domestic chicken (red jungle fowl), and two more distantly related species (grey and green jungle fowl) were studied. All *Gallus* species harbored this class of elements, although the different lines of domestic chickens and different species of jungle fowl bore distinguishable complements of the proviral loci. Jungle fowl appeared to have fewer copies than domestic chickens. For three randomly isolated proviral loci, domestic chickens (*G. gallus* subsp. *domesticus*) and red jungle fowl (*G. gallus* subsp. *gallus*) showed only a proviral state, whereas the most primitive and divergent of the jungle fowl, the green jungle fowl (*G. varius*), consistently demonstrated only preintegration states or disparate alleles. The presence of this family in all *Gallus* species and of related sequences in other genera suggests that a primordial founding integration event occurred prior to the evolutionary separation of *Gallus* species and possibly related genera. Additionally, at least one proviral locus has been acquired subsequent to speciation, indicating that this family was actively infectious after the primary founding event. This conserved, repetitive proviral family appears to represent the vestigial remnant of an avian retrovirus class related to and evolutionarily more ancient than the Rous-associated virus-0 family of avian endogenous retroviruses.

Endogenous retroviruses are found as moderately repetitive elements, associated with many avian genera (6, 8, 17, 18, 52), and the Rous-associated virus-0 (RAV-0) endogenous retroviruses found in the genus *Gallus* are perhaps the best studied (35). Members of the RAV-0 family are related to exogenous avian sarcoma-leukosis viruses (ASLVs) (37) but, in contrast to ASLVs, are not generally associated with pathogenesis (10, 29). At least 26 distinct RAV-0 loci have been genetically characterized in the domestic chicken genome by segregation analysis during genetic crosses (35). These loci represent distinct phenotypes, including a variety of partially defective proviruses, proviruses which are capable of producing infectious virions, and proviruses which are associated with alterations in host gene function (2, 18, 35, 55).

Genetic and phenotypic evidence for the presence of RAV-0 elements only in red jungle fowl (*Gallus gallus* subsp. *gallus*), the progenitor to the domestic chicken (*G. gallus* subsp. domesticus), and in ringneck pheasant (*Phasianus cholcicus*), representing a different genus, and the absence of RAV-0 in jungle fowl closely related to *G. gallus*, such as the grey jungle fowl (*G. sonnerati*), green jungle fowl (*G. varius*), and Ceylonese jungle fowl (*G. lafayetti*), establishes that the genetic distribution of RAV-0 does not follow phylogenetic relationships (14, 15, 17). Therefore, RAV-0 entered chickens and ringneck pheasants by separate infections after speciation but prior to domestication of chickens and have been maintained in these species by independent, infrequent germ line infections.

* Corresponding author.
† Present address: Cetus Corp., Emeryville, Calif.

We have recently described the molecular characterization of several independent and unique members from a new family of endogenous retroviruslike elements (EAV-0 family) cloned from the line-0 chicken; line-0 lacks RAV-0 elements (11, 12). This family, related to the Rous sarcoma-avian leukosis virus family, has subsequently been confirmed by other independent researchers (7). This novel family (EAV-0), distinguishable by short long terminal repeats (LTRs) (5a), is present in not less than 50 copies per haploid genome per individual, arising perhaps through multiple independent germ line insertional events, gene duplication events, or combinations of both events.

We have used Southern blot analysis of genomic DNA and locus-specific probes to determine the haploid frequency and distribution of several unique loci of this novel family in eight inbred lines of domestic chickens, three species of jungle fowl representing the genus *Gallus*, and several other avian genera. The phylogenetic distribution of EAV-0 is distinct from the distribution of RAV-0. Based on our analysis, EAV-0 represents a more conserved and more ancient retrovirus family which entered *Gallus* spp. prior to speciation and perhaps prior to the evolutionary divergence of other avian genera. Additionally, one proviral locus studied here appears to have been acquired subsequent to the separation of the green jungle fowl from the taxonomic line leading to domestic chickens.

MATERIALS AND METHODS

Cloning and subcloning of EAV-0 elements. All EAV-0 clones were isolated from the Line-0 chicken genomic DNA libraries as described previously (12).

Generation of locus-specific probes and preparation of radioactive probes. Based upon the fine restriction site map-

ping of viral genes and LTRs within each EAV clone studied, probes flanking the EAV elements were selected. For probing, DNA restriction fragments representing only flanking DNA were isolated following electrophoretic separation through low-melting-point agarose (54). Alternatively, each fragment within the gel slice was labeled without further purification to a specific activity of $\geq 5 \times 10^8$ cpm/μg by incorporation of [^{32}P]dCTP with a random primer method (13). Each fragment was confirmed to identify only its respective *Eco*RI restriction fragment by Southern blot analysis of DNA from the original lambda clone. Probes identifying a single predicted DNA junction fragment or a fragment consistent with a preintegration site in genomic digests were considered locus specific. Junction fragments corresponding to specific loci were visualized by Southern blot analysis following digestion at either the highly conserved internal *Eco*RI sites within the putative *pol* gene or at a unique *Bam*HI site positioned in the leader sequence upstream of the start codon of the putative p19 gene, allowing identification of 5′ and 3′ junction fragments, respectively.

Sources of avian samples and preparation of high-molecular-weight genomic DNAs. Blood samples from *G. gallus* subsp. *domesticus* from line 11, line 22, and line 77 were acquired from Spafas, Inc. Line 11 has been an isolated line for 28 years, whereas line 22, originating in Germany, has been maintained by Spafas for 12 years. Blood samples of line-0 (RAV-0$^-$), line 6_3, line 7_2, line $I15_4$, and line $I15_5$ and DNA from *G. sonnerati* (grey jungle fowl) were generous gifts from Lyman Crittenden, U.S. Department of Agriculture Regional Poultry Research Center at East Lansing, Mich. These lines were established in 1945, with the sublines $I15_4$ and $I15_5$ isolated in 1967 (L. Crittenden, personal communication). Blood samples of *G. varius* (green jungle fowl) were the generous gifts from two zoos. Most *G. varius* in U.S. zoos today are proposed to be related to birds imported by a consortium of pheasant breeders in 1976. Mary Healy and Jay Lauver at the San Antonio Zoo in San Antonio, Tex., provided samples from 13 siblings arising from a cross of a male originating from a New York breeder to a female originating from a California breeder. Alan A. Lieberman, Amy L. Shima, and Don Janssen at the San Diego Zoo, San Diego, Calif., provided samples from two birds in their collection which they indicated are seropositive for reticuloendotheliosis virus (REV).

DNA was isolated from heparinized frozen whole blood in samples of 100 to 200 μl by using minor modifications to a deproteination procedure described by Hughes et al. (17). Saline washes were incorporated during processing of frozen blood to remove cytoplasmic proteins and hemoglobin released during the freeze-thaw lysis. Never more than 200 μl of starting whole blood was processed in the final sodium dodecyl sulfate (SDS)-pronase digestion solution volume of less than 5 ml. The samples were treated with RNase A and subjected to another round of deproteination.

Restriction enzyme digestions and Southern blotting. DNA was digested under the buffer conditions recommended by the manufacturers. Digestions were stopped by addition of excess EDTA (≥10 mM final) and SDS (0.1% final) in a Ficoll-based gel loading buffer. DNA digestions stabilized in a Ficoll-based gel loading buffer (0.1% SDS, >10 mm EDTA) could be stored at −20°C for extended periods (up to 4 years) with no apparent evidence of degradation.

Genomic DNA restriction fragments were resolved by electrophoresis through 0.8% agarose gels in Peacock buffer (31). DNA was transferred to the nylon-based membrane ZetaBind by rapid alkaline transfer including a mild acid depurination (32). Solid-phase nucleic acid hybridization under highly stringent conditions with 50% formamide at 42°C was done with heparin as the sole blocking agent (39). For low-stringency hybridizations (16), a standard Southern hybridization with Denhardt hybridization solutions and heterologous (salmon sperm) DNA as a blocking agent was used (26, 42). Washing conditions followed guidelines outlined by Meinkoth and Wahl (26). Prior to reprobing, existing probe was removed by washing with two cycles of 90 to 95°C washes with 0.1× standard saline phosphate citrate–0.1% SDS for 15 minutes as outlined by Meinkoth and Wahl (26), followed by a 37°C treatment in 0.4 N NaOH for 30 min, and finally neutralized to pH 7.5 and equilibrated to either 0.1× SSPC–0.1% SDS for storage or to prehybridization buffer for immediate reprobing (26).

RESULTS

EAV-0 elements are found in all three *Gallus* species. We have previously demonstrated the presence of numerous copies of the EAV-0 family of proviruses in the genome of domestic chickens bred free of RAV-0, but had not yet extended our study to survey the frequency and diversity of elements among different inbred lines and other species. In order to distinguish between RAV-0-related sequences and EAV-0 sequences in various inbred lines and other avian species, it was first necessary to probe genomic DNA under conditions defined as high stringency. Southern blotting of genomic DNA digested with *Eco*RI will identify the 5′ junction fragment of each EAV-0 locus or will detect RAV-0 elements with probes at high-stringency conditions derived from the *gag* gene of EAV-0 and Rous sarcoma virus (RSV), respectively.

DNA samples from three domestic lines (line-0, line 11, and line 7_2) and from three jungle fowl species were hybridized with RSV (Fig. 1A) and EAV-0 (Fig. 1B) *gag*-specific probes. Spafas lines 7_2 and 11, the parental lines used in the development of the RAV-0-free line line-0, and the single red jungle fowl contained RAV-0 elements, as reported previously (1). Line 7_2 carried *ev-1* and *ev-2*, and line 11 carried the single defective provirus locus *ev-1*. Line-0 and *G. sonnerati* were free of detectable RAV-0 elements upon probing with an RSV probe at high-stringency conditions. On the other hand, *G. varius* appeared to contain RAV-0 proviruses, in contrast to reports describing the lack of phenotypic evidence for RAV-0 in feral jungle fowl (53).

The birds from the San Diego zoo analyzed in this study were not related to each other and demonstrated different numbers of loci. The 13 birds from the San Antonio zoo are siblings with one parent from a California breeder. It is conceivable that the San Antonio birds are related to the San Diego female, since 50% of them had four of five loci in common with the San Diego female. The San Antonio birds showed two loci at 5 and 10 kilobases (kb) which failed to segregate suggesting homozygosity at these loci. The other loci segregated independently, and none of the elements appeared to be sex linked.

The EAV-0 *gag* probe detected numerous bands representing different 5′ junction fragments for each proviral locus of the EAV-0 family. EAV-0 sequences were detected in line-0 and *G. sonnerati*, both of which lacked detectable RAV-0 elements. Restriction enzyme digestion patterns showed minor differences between the two parental strains of line-0. Complex patterns of bands with subtle distinctions were observed in *Eco*RI digests of genomic DNA from the

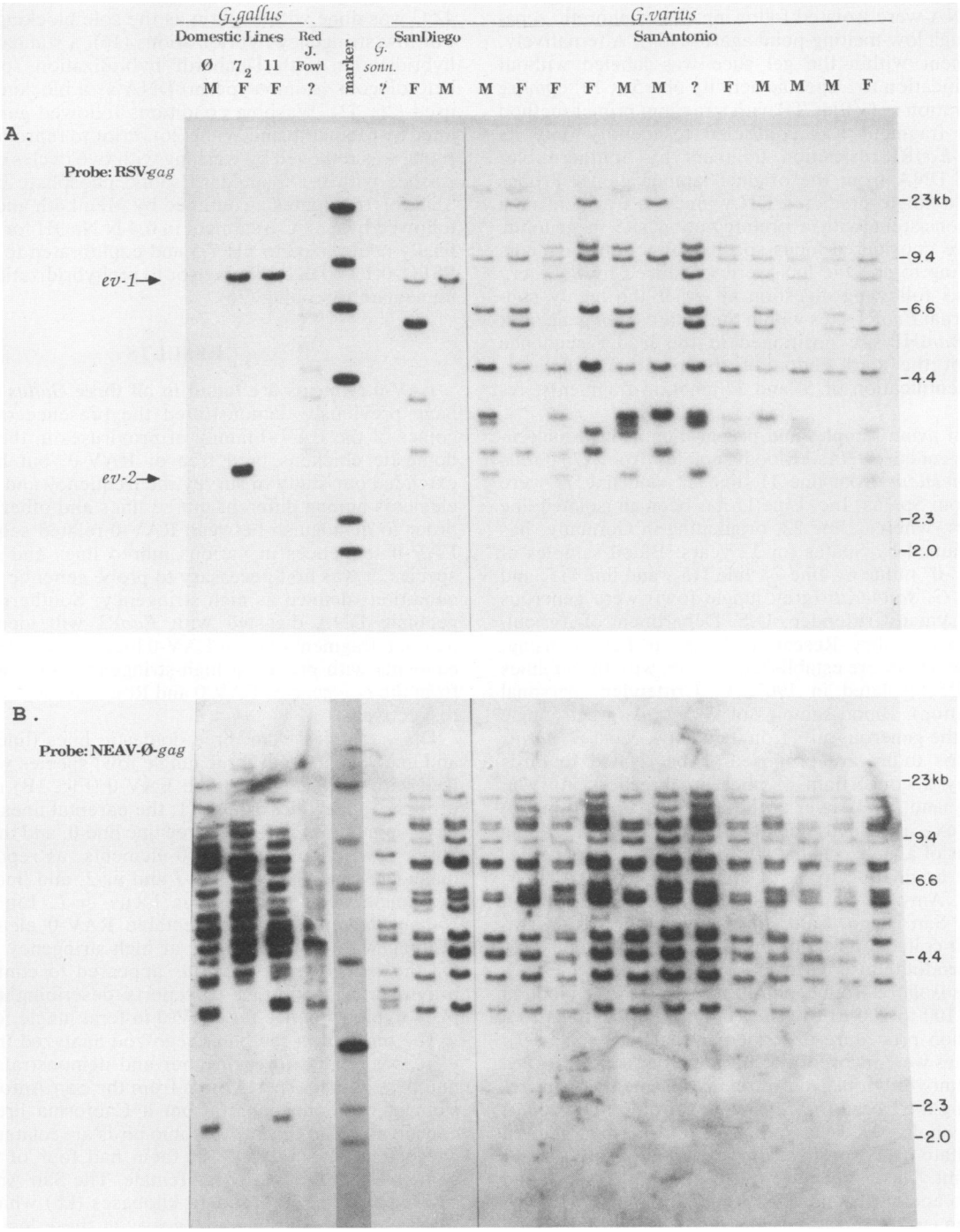


FIG. 1. Determination of RAV-0- (A) and EAV-0 (B)-related sequences in domestic chickens and three species of jungle fowl by Southern blotting analysis. *Eco*RI-digested genomic DNAs (2.5 μg per lane) were electrophoresed through 0.8% agarose and transferred to ZetaBind membranes with an alkaki transfer technique. Hybridization followed high-stringency conditions, with heparin as the sole blocking agent. The probes covered the *gag* regions. The 1.29-kb *Pst*I fragment from pATV8 RSV includes 36 bp of untranslated leader sequence, the p19, p10, and half of the p27 genes. The EAV-0 probe *Bam*HI-*Xho*I 750-bp fragment from clone 47.1 includes sequences similar to half of p10 and p27. The molecular size markers are end-labeled lambda DNA digested with *Hin*dIII. The marker lane includes the highly conserved 3-kb *Bam*HI-*Eco*RI EAV-0 *gag*-reactive internal fragment from clone gp11 as a hybridization control. Line-0 is the RAV-0-free line bred from parental lines 11 and 7_2. The sex of each chicken is indicated above the lanes. J., Jungle; G. sonn. *G. sonnerati*.

eight different lines of domestic chickens, whereas the patterns were remarkably constant within each inbred line (data not shown). Red jungle fowl shared many loci with domestic chicken. *G. sonnerati* and *G. varius*, while sharing some bands with domestic chickens, showed patterns that were distinctive in the number, position, and intensity of bands from each other and from domestic chicken DNA. For example, DNA from *G. varius* showed a cluster of three bands between 3 and 4 kb which was absent in *G. sonnerati*. The *G. varius* samples from the two sources resembled each other more than did those from other *Gallus* species. Jungle fowl also appeared to have fewer and a less heterogeneous array of junction fragments than domestic chickens.

Sequences flanking proviral loci contain distinct repetitive DNA. In order to develop locus-specific probes for the study of the haploid frequencies of separate proviruses, radiolabeled probes were generated from DNA restriction fragments located 5′ to the LTRs of four independent clones isolated in low-melting-point agarose. Restriction maps of the 5′ *gag*-specific *Eco*RI junction fragments from four EAV-0 independent clones are shown in Fig. 2A. Each clone represented an independent locus, since each had a different-size *Eco*RI junction fragment, with restriction site differences residing in the upstream flanking sequences. Restriction mapping also revealed strong conservation (greater than 90%) for internally located viral *Bam*HI, *Xho*I, and *Eco*RI sites, which coincided with fragments observed in genomic mapping studies. The additional *Bam*HI site found in clone 47.1.1 in the middle of a putative p10 gene was not an artifact of cloning but represented a single-copy allele, since a 750-base-pair (bp) restriction fragment was detected at a single-copy level in genomic digestions with *Bam*HI (data not shown).

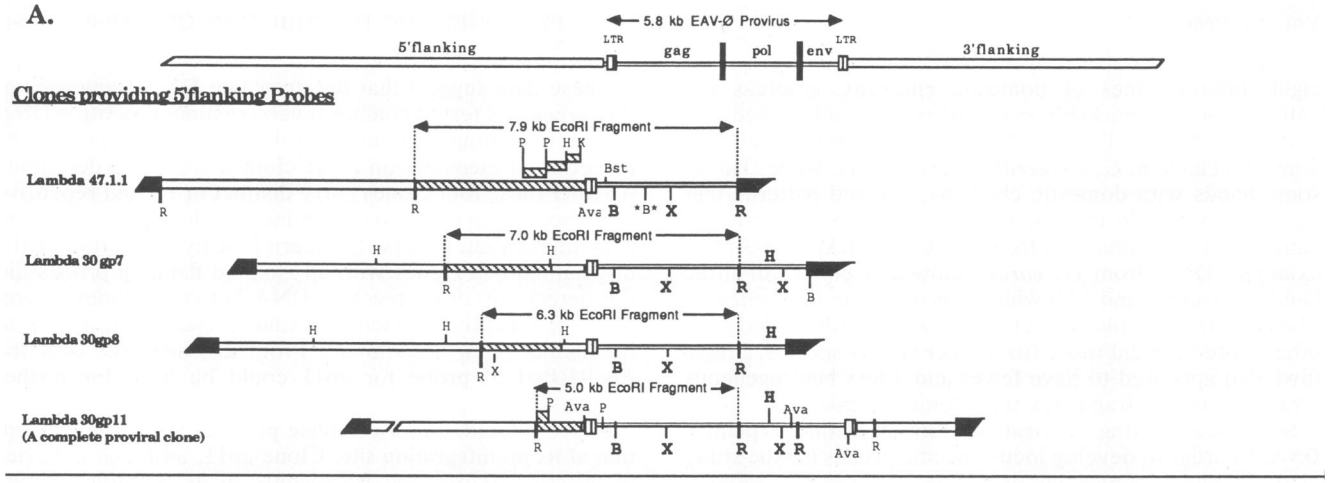
Molecular characterization and mapping of conserved internal restriction sites in several different EAV-0 clones indicated that the 5′ junction fragments for each corresponding locus should be distinguishable in genomic *Eco*RI digestions when probed with 5′-flanking probes. Similarly, 3′ proviral junction fragments should be identifiable following digestion at the single conserved *Bam*HI site located immediately upstream from the putative start codon for the p19 gene when probed with a 3′-flanking probe. Multiple DNA fragments were observed when *Eco*RI-digested genomic DNA was probed with separate flanking fragments (Fig. 2B). Clone 47.1.1 apparently contained repetitive DNA across 1.5 kb, since three additional proximal fragments from this clone also failed to be locus specific, although the most distal sequences have not yet been tested. The *Eco*RI-*Hin*dIII probe of clone gp7 detected a smear of bands, whereas probes of other clones detected a more limited array of hybridizing bands. The *Eco*RI-*Ava*I probe of clone gp11 contained a portion (23 bp) of U3 including all of the IR3, and although the probe could theoretically detect an LTR-containing fragment, the amount of sequence was not enough to drive significant hybridization to the 3′ junction fragment of cloned proviruses under the conditions used in genomic blotting (data not shown). This probe detected multiple bands in genomic DNA (Fig. 2B, probe B), again suggesting that it contains a repetitive sequence. In addition, it also detected a strongly hybridizing fragment of 5 kb, which is the expected size for the junction fragment. A smaller *Eco*RI-*Pst*I probe (Fig. 2B, probe A), located more distal from the provirus, detected only the expected 5-kb fragment and was proposed to be locus specific. The more proximal sequences between the *Pst*I site and the *Ava*I site must therefore contain repetitive sequences.

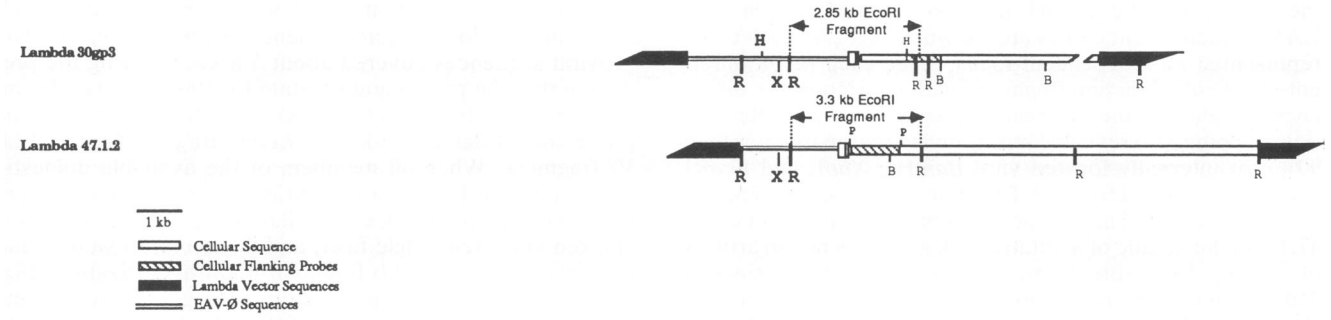
These data suggest that the upstream DNA sequences of the proviruses tested contain several distinct repetitive DNA motifs. By testing the flanking probes from each clone for the detection of elements on other clones, we could determine whether these four clones carry distinct or related repetitive sequences in an effort to determine whether these represent gene duplication events. Southern blot hybridization of the original lambda clones with all isolated flanking probes did not detect any cross-reactive DNA between clones; therefore, the repetitive sequences found on each clone appear to be distinct (data not shown). From this analysis, only the *Eco*RI-*Pst*I 5′ probe for gp11 could be used for further analysis.

Characterization of a complete proviral locus and description of its preintegration site. Clone gp11, although it carries many stop codons and frameshifts in its putative reading frames and has a major deletion in the putative *env* gene, is a complete provirus in the sense that viral gene sequences reside between two LTRs (unpublished data). The complete provirus clone has been mapped, and the boundaries of both upstream and downstream sequences were determined. The proviral sequences covered about 5.8 kb, allowing the prediction that the preintegration state for this locus resides on a 2.3-kb *Eco*RI fragment (Fig. 3A). Therefore, the 5′-flanking probe should detect a 5-kb V^+ *Eco*RI fragment or a 2.3-kb V^0 fragment. When all members of the available domestic inbred chicken lines and the various jungle fowl were analyzed with the locus-specific 5′-flanking probe, all domestic chicken lines, red jungle fowl, and *G. sonnerati* were found to contain only the 5-kb fragment upon hybridization (Fig. 3B). All *G. varius* samples exhibited a 2.3-kb hybridizing fragment consistent with the predicted preintegration state (V^0). This probe failed to detect any hybridization at high-stringency conditions in genera other than *Gallus*, suggesting that this probe may also be genus specific (data not shown).

Analysis of two proviral loci with 3′-flanking DNA probes. Locus-specific probes from the 3′-flanking sequences of the partial proviral clones 47.1.2 and gp3 (Fig. 2B) were used for integration site analysis. A unique 2.85-kb *Eco*RI 3′ junction fragment in clone gp3 was detectable by hybridization with a 930-bp *Hin*dIII-*Bam*HI 3′-flanking probe (Fig. 4A). Hybridization of *Eco*RI-digested samples revealed the 2.85-kb (V^+) band in domestic chickens, *G. gallus*, and *G. sonnerati* (Fig. 4B). The smaller reactive band of 575 bp was due to a second *Eco*RI site present in both the probe and the hybridizing region. These findings suggest homozygosity for this locus in all samples. *G. sonnerati* exhibited a smaller fragment, representing a second allele, in addition to the expected V^+ allele. *G. varius* from both zoos showed a larger fragment while carrying the conserved 575-bp fragment, indicating that a restriction site polymorphism is located 5′ to the probe in *G. varius*. It cannot be determined from this analysis whether the larger fragment represents the preintegration state for this locus.

The map of the lambda clone 47.1.2 identifying a second locus shows the location of the flanking probe as well as its 3.4-kb 3′ *Eco*RI junction fragment and the fragment representing the predicted preintegration site (Fig. 5A). Since clone 47.1.2 does not contain the upstream portion of the provirus, the size of the *Bam*HI proviral junction fragment could only be predicted if the highly conserved *Bam*HI site in *gag* is present in the actual locus. The size of the predicted V^+ *Bam*HI fragment would be 7.3 kb. The Southern blot of *Eco*RI and *Bam*HI digests of seven DNAs representing five lines of domestic chickens revealed three genotypes comprising two independently segregating allelic loci (Fig. 5B):


A.
5.8 kb EAV-Ø Provirus
5'flanking
LTR
gag
pol
env
LTR
3'flanking
Clones providing 5'flanking Probes
7.9 kb EcoRI Fragment
Lambda 47.1.1
Bst
7.0 kb EcoRI Fragment
Lambda 30 gp7
6.3 kb EcoRI Fragment
Lambda 30gp8
5.0 kb EcoRI Fragment
Lambda 30gp11
(A complete proviral clone)


Clones providing 3'flanking Probes
Lambda 30gp3
2.85 kb EcoRI
Fragment
Lambda 47.1.2
3.3 kb EcoRI
Fragment
1 kb
Cellular Sequence
Cellular Flanking Probes
Lambda Vector Sequences
EAV-Ø Sequences

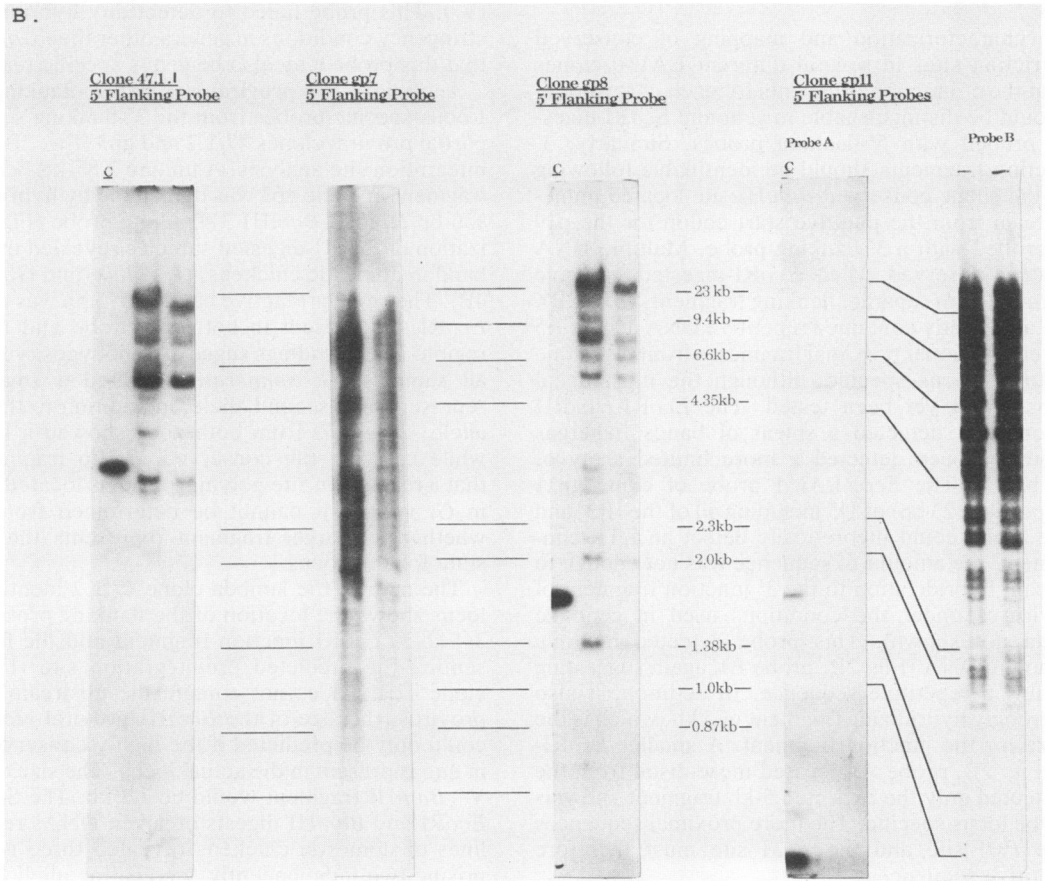

B.
Clone 47.1.1
5' Flanking Probe
Clone gp7
5' Flanking Probe
Clone gp8
5' Flanking Probe
Clone gp11
5' Flanking Probes
Probe A
Probe B
C
23 kb
9.4kb
6.6kb
4.35kb
2.3kb
2.0kb
1.38kb
1.0kb
0.87kb

FIG. 2. Development of locus-specific probes. (A) Restriction maps and diagrams of putative coding sequences of four independently isolated clones providing 5'-flanking (upper panel) and 3'-flanking (lower panel) probes. The consensus structure for the EAV-0 provirus is shown aligned above the EAV-0 clones. Vector sequences are represented by solid boxes. The double line indicates the viral sequences, and the open boxes correspond to cellular sequences. The hatched boxes represent fragments used as flanking probes in panel B. The size of each *Eco*RI junction fragment is indicated above the map of the clone. Restriction sites: R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; K, *Kpn*I. The conserved *Bam*HI, *Xho*I, and *Eco*RI sites are shown by the larger letters. Additionally, only the *Pst*I and *Ava*I sites which are significant to the generation of the probes are shown, and other sites existing within the provirus are not shown. (B) Southern blot analysis with probes from the four clones providing 5'-flanking sequences. Lanes C represent markers, which included a hybridization control fragment specific for each locus. Genomic DNAs (2.5 μg) were digested with *Eco*RI, and the first lane on the left is a sample of line-0, and the other is a sample of line 11. Probes used were as follows: 47.1.1, *Eco*RI-*Ava*I; gp7, *Eco*RI-*Hin*dIII; gp8, *Eco*RI-*Hin*dIII; gp11, *Eco*RI-*Pst*I (A) and *Eco*RI-*Ava*I (B).

two homozygous states (V^N/V^N and V^+/V^+) and one heterozygous state (V^N/V^+). The first line-0 individual tested appeared to be heterozygous in both digests, whereas the second line-0 individual carried only the 7.3-kb *Bam*HI fragment and the corresponding 3.3-kb *Eco*RI fragment.

Haplotype analysis of the jungle fowl samples for the presence of the provirus 47.1.2 (Fig. 5C) revealed that the single red jungle fowl was heterozygous and the single *G. sonnerati* sample was homozygous (V^N). The *G. varius* samples all demonstrated a hybridizing band of 3 kb which was different from either of the characterized states and represented a restriction site polymorphism in either the cellular sequence or the provirus. The inability to detect the other alleles may be related to a limited sample size or a founder effect as a result of speciation.

Analysis of a solo LTR reveals evidence consistent with the existence of a solo LTR and its preintegration site but fails to give evidence for the presence of a complete provirus. One clone was determined to be a solo LTR by hybridization and sequence analysis. This solo LTR locus resided on a 6.3-kb *Eco*RI fragment (Fig. 6A). An *Xba*I-*Hin*dIII fragment was subcloned for fine mapping, and the LTR was further restricted to a 1.2-kb *Sac*I-*Acc*I fragment (Fig. 6A). Molecular characterization and analysis of expression from this locus are in progress.

A unique 5'-flanking locus-specific probe of 2.4 kb was used to identify this locus in genomic digests of DNA from domestic chickens. Comparison of the solo LTR locus restriction map with the consensus restriction map for the EAV-0 provirus family along with the orientation of the LTR allowed predictions to be made about possible alternative states for this locus (Fig. 6A). If a complete provirus were present at this locus, then this flanking probe should detect an *Eco*RI junction fragment of 8 kb. Analogously, the corresponding preintegration site should be represented by an *Eco*RI restriction fragment of approximately 6.0 kb.

The collection of avian genomic DNAs was surveyed by Southern blot analysis for the existence of the three possible alternative states (V^+, V^0, or solo LTR). Only a restriction fragment of 6.3 kb was found in all lines of domestic chickens, suggesting the presence of the solo LTR. *G. varius* and *G. sonnerati* appeared to have reactive fragments migrating with slightly lower molecular weights than those observed in the DNAs from domestic chickens. The single sample of *G. sonnerati* demonstrated a band with the fastest mobility. This slight difference in mobility was observed in several studies, suggesting that a preintegrative state exists; however, the 246-bp difference between the solo LTR state and the preintegration state could not be resolved with certainty in most Southern blots due to a combination of variability in resolution during electrophoretic separation of genomic DNA digests and artifacts introduced during the DNA transfer from a pliable gel matrix to a flexible nylon membrane.

To specifically address this issue, the two DNAs with apparently different mobilities were mixed together and run in a single track between two tracks containing each respective DNA alone. This analysis revealed that the band in *G. sonnerati* did migrate faster than the band observed in the DNA of line-0 (Fig. 6B), consistent with a difference of about 200 to 300 bp. *G. varius* also contained a restriction fragment with a faster mobility than the band in domestic chicken lines 7_2, 6_3, line-0, and 11. Therefore, *G. sonnerati* and *G. varius* contain only the preintegration state for this locus. None of the samples tested appeared to contain a V^+ locus.

Table 1 summarizes the implied haploid frequencies among the jungle fowl species and domestic lines for all loci examined. Three loci (gp11, gp3, and the solo LTR) appeared to have only one allelic type in domestic chickens and the less divergent jungle fowl, *G. sonnerati* and *G. gallus*, while the green jungle fowl showed different restriction fragments for these loci. Only one (47.1.2) of the four loci examined showed allelism in domestic chickens and red jungle fowl. The green jungle fowl *G. varius* again showed a distinctive restriction fragment not seen in other chickens. Lines 7_2 and 77 were homozygous for the preintegrative locus (V^0/V^0), whereas line 6_3 was homozygous for the viral state (V^+/V^+). Lines $I15_4$ and $I15_5$ showed a low haplotype frequency for the V^+ state (1 of 20), and line 22 had a haplotype frequency for the V^0 state of 2 of 12. Line 11 and line-0 had a haplotype ratio for V^+ to V^0 of 10:6 and 16:12, respectively.

TABLE 1. Haplotype frequencies of EAV-0 loci[a]

Chicken line	No. tested	No. of loci with indicated haplotype									
		gp11			Solo LTR			gp3		47.1.2	
		V^+	V^0	N	V^+	V^0	Solo LTR	V^+	N	V^+	N
Domestic chickens											
White Leghorn											
Line-0	14	28	0	0	0	0	28	28	0	16	12
11	8	16	0	0	0	0	16	16	0	10	6
22	6	12	0	0	0	0	12	12	0	10	2
7_2	5	10	0	0	0	0	10	10	0	0	10
6_3	5	10	0	0	0	0	10	10	0	10	0
$I15_4$	5	10	0	0	0	0	10	10	0	1	9
$I15_5$	5	10	0	0	0	0	10	10	0	0	10
Rhode Island Red 77	6	12	0	0	0	0	12	12	0	0	12
Jungle fowl											
G. gallus	1	2	0	0	0	0	0	2	0	1	1
G. sonnerati	1	2	0	0	0	2	0	1	1	2	0
G. varius	14	0	28	0	0	28	0	0	28	0	28

[a] The appearance of a single restriction fragment was assumed to represent a diploid homozygous state.

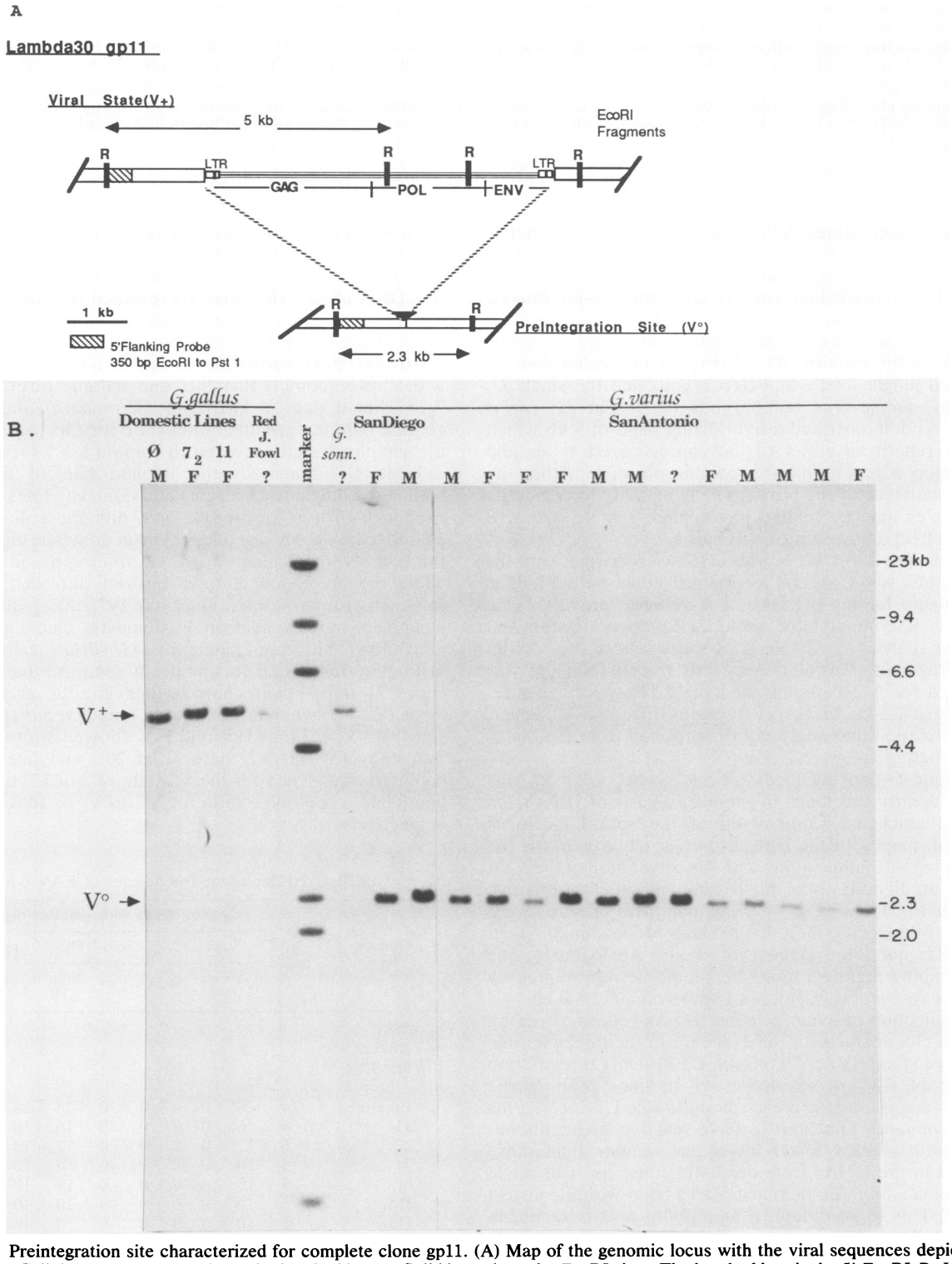


FIG. 3. Preintegration site characterized for complete clone gp11. (A) Map of the genomic locus with the viral sequences depicted with a triple line. Cellular sequences are shown by hatched boxes. Solid bars show the *Eco*RI sites. The hatched box is the 5′ *Eco*RI-*Pst*I fragment of 350 bp. The predicted preintegration site is shown and resides on a 2.3-kb *Eco*RI fragment. (B) Southern blot of three domestic lines of chickens and three species of jungle fowl, each digested with *Eco*RI (2.5 μg per lane). The marker lane contains ^{32}P-end-labeled lambda *Hin*dIII fragments. The virus-containing locus is indicated by the V^+, and the preintegration site is shown by V^0. See also Fig. 1 legend.

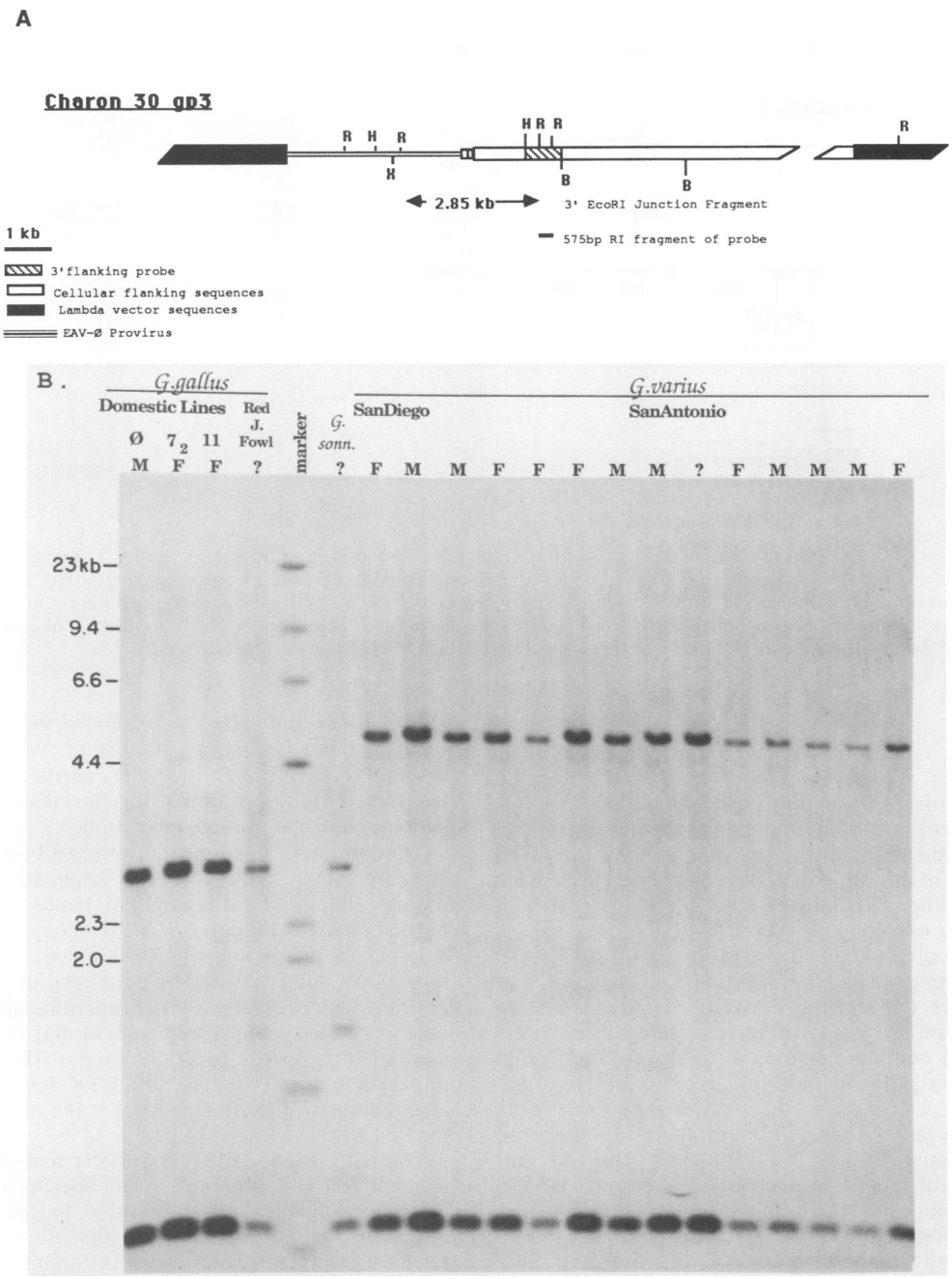


FIG. 4. Analysis of Charon 30 gp3 loci. (A) Map of the lambda clone, with the major *Eco*RI fragments shown. The open boxes depict cellular flanking seqences, and the solid boxes are the lambda sequences. The hatched box represents the 3′-flanking probe. The lower map depicts the predicted preintegration state. Note that the *Hin*dIII-*Bam*HI probe contains an internal 575-bp *Eco*RI fragment, seen in all the samples tested in panel B. R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI. (B) Southern blot analysis with 2.5 μg of *Eco*RI-digested DNA from three domestic lines and three species of jungle (J.) fowl. See also Fig. 1 legend.

EAV-0 sequences related to the putative p27 gene detect reactive restriction fragments in divergent genera. Another family of endogenous retroviruses related to RSV has been described for the Japanese quail (7). In order to determine whether EAV-0 probes detect similar sequences in quail and other genera, an EAV-0 *gag* subgenomic probe containing the putative p27 capsid gene was used to probe genomic DNAs from other genera (Fig. 7). This probe detected a distinct band in the Japanese quail DNA but not in DNAs from other avian genera, which increased in intensity when the hybridization stringency was lowered. The RSV probe did not as readily detect this band even at lowered stringency or with extended exposure. The subgenomic EAV-0 *gag* probe bearing the p19 coding domain failed to detect a defined band when the same blot was reprobed. Probes consisting of either *pol*- or *env*-related sequences from EAV-0 detected reactive DNA in other species only with lowered stringency conditions, allowing for up to 30% base pair mismatches (16).

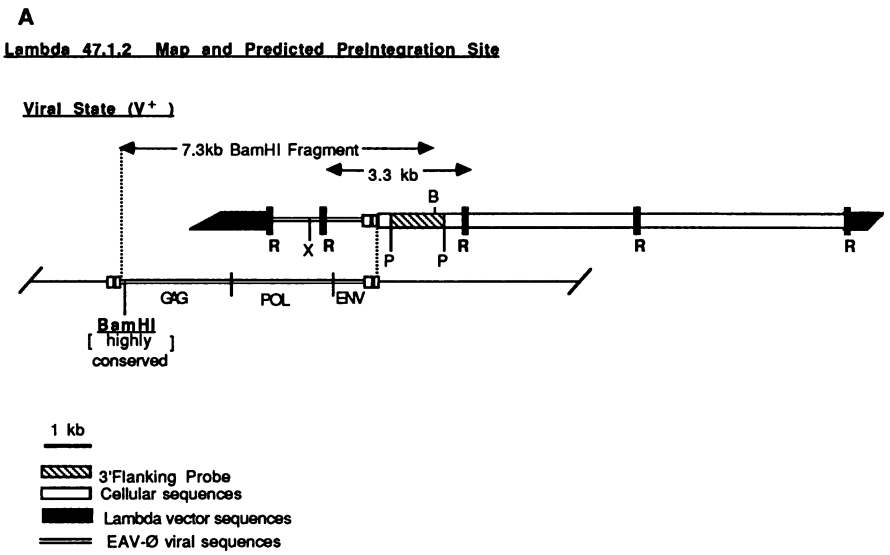


FIG. 5. Lambda 47.1.2. (A) Map of the lambda clone, with the major *Eco*RI and *Bam*HI fragments shown. The open boxes depict cellular flanking sequences, and the solid boxes are the lambda sequences. The hatched box represents the 3′-flanking probe. R, *Eco*RI; X, *Xho*I; P, *Pst*I; B, *Bam*HI. (B) Southern blot analysis of DNAs with the enzymes *Eco*RI and *Bam*HI. DNAs from the different lines are labeled (2.5 μg per lane). The virus-containing locus is indicated by the V^+, and the variant locus is marked V^N. (C) Southern blot analysis with 2.5 μg of *Eco*RI-digested DNA from three domestic lines and three species of jungle fowl. See also Fig. 1 legend.

DISCUSSION

To follow the evolution of a new family of endogenous avian retroviruses, we surveyed for the presence of specific proviruses in birds representing inbred lines of *G. gallus* (domestic chicken) and then extended the study to related *Gallus* species and different genera. The general approach has been applied to the study of endogenous proviruses from avian (14, 15), feline (3), murine (24, 28, 30, 45, 47), and primate (4, 5, 38, 44) species.

The data presented establish the presence of the EAV-0 family in divergent species, supporting the proposal that EAV-0 family is an ancient retroviral family predating RAV-0. Line-0 and *G. sonnerati*, lacking detectable RAV-0 elements, clearly contain elements of this new family (Fig. 1). While *G. varius*, the most dissimilar and primitive of the jungle fowl (21), also harbors the EAV-0 sequences in its genome, it consistently shows different alleles upon examination of individual EAV-0 loci (Table 1). The different *Gallus* species and inbred lines clearly share some EAV-0 junction fragments. The inbred lines minimally represent eight random isolates from a theoretical primordial population; therefore, the limited variability that they do show indicates that a high degree of positional conservation existed in the original gene pool, and the minor differences most probably represent a founder effect due to inbreeding rather than recent transpositions. For example, at the 47.1.2 locus, line 7_2 was homozygous for the preintegration state (V^0), whereas line 6_3 was homozygous for the viral state (V^+) and line-0 and line 11 showed allelism at this locus.

The analysis of the organization of the four loci among the domestic lines and the wild jungle fowl species indicates the order of provirus acquisition during speciation. *G. varius* is V^0 for two loci (gp11 and solo LTR) and shows novel polymorphisms for the two loci characterized by their 3′-flanking probes (gp3 and 47.1.2). *G. sonnerati* is also V^0 for the solo LTR; however, unlike *G. varius*, it was V^+ for gp3 and gp11. While the ubiquity of this family in the genus *Gallus* is consistent with its presence before speciation, the variability seen for the loci examined indicates that none of them infected *Gallus* spp. before *G. varius* diverged. The distinctive alleles seen in the *G. varius* samples may have been fixed in the genome of this species as a result of early geographic isolation and speciation.

The finding of apparent homozygosity at the V^+ loci for some species tested has several implications. (i) Proviruses did not integrate into essential genes or seriously alter normal biological functions. (ii) Homozygosity may be coincidental to inbreeding during domestication. (iii) The homozygous genotype may be beneficial to the species. Integrations which affect essential functions may be maintained only as heterozygotes (20, 41, 43) or may undergo rearrangements which restore function, such as the dilute coat color mutation in mice (48). The solo LTR described here may be an example of the latter, since it is the only detectable state for that locus.

The data provided herein suggest that the EAV-0 family has entered the different avian species as a result of a prespeciation event in addition to independent horizontal infections for the following reasons. First, the pattern of 5′ junction fragments reveals bands common to all species of jungle fowl and domestic chickens, suggestive of prespeciation events. Second, since germ line integrations occur randomly and infrequently, it is unlikely that they would occur at the same genetic location. In fact, our data indicate that at least some elements became fixed after the green jungle fowl (*G. varius*) split from the evolutionary line leading to domestic chicken.

As a class of elements, EAV-0 appears to have entered the genus *Gallus* earlier than RAV-0 (Fig. 8). The presence of similar vestigial sequences in other genera may be markers with which to trace evolutionary relationships. Cytogenetic studies of eight species of the order *Galliformes* establishes two groups, the first consisting of *Coturnix*, *Gallus*, *Numida*, and *Pavo*, which are related but clearly distinct lines, and the second consisting of *Phasianus* and closely related New World species derived from it (46). We observed $p27^{gag}$-

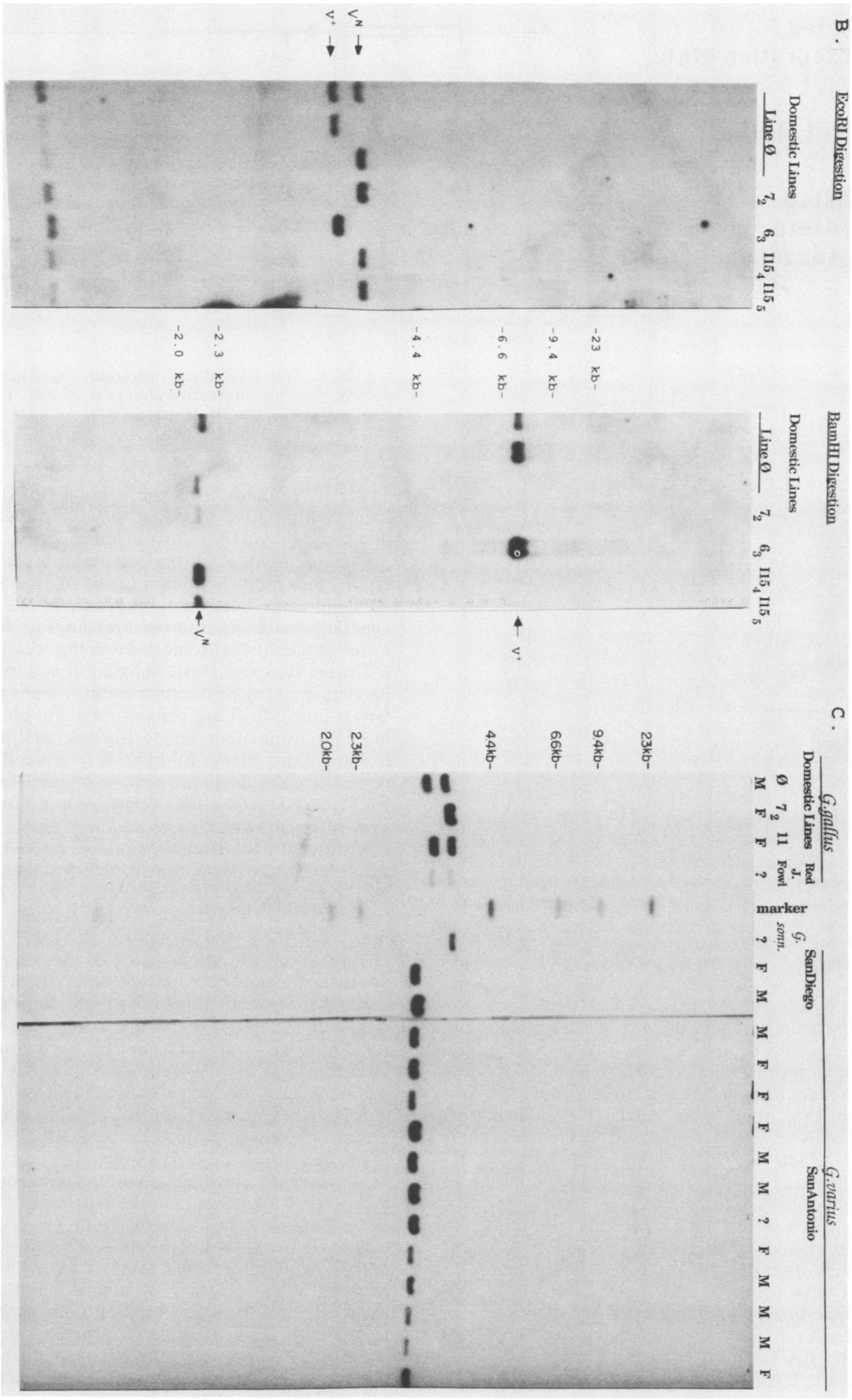
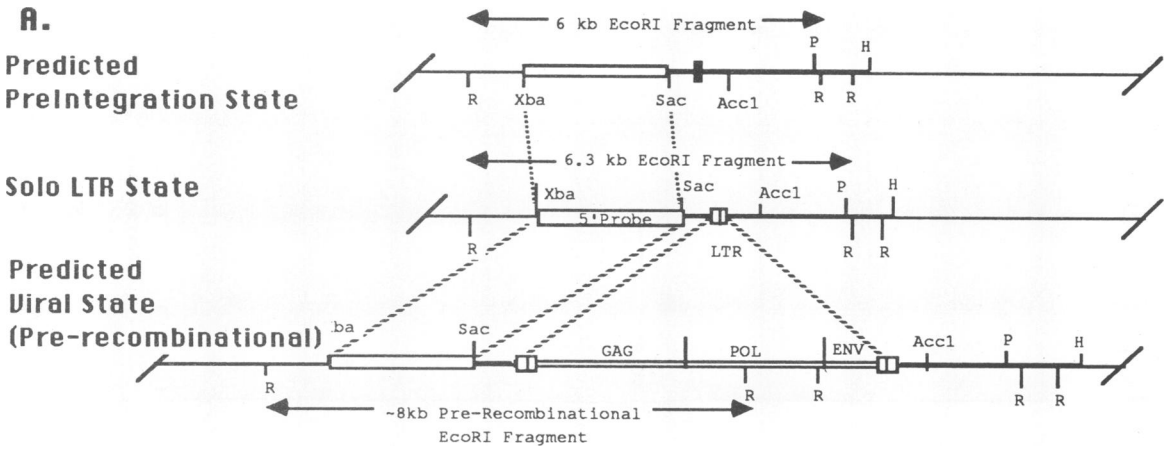


FIG. 5—*Continued.*



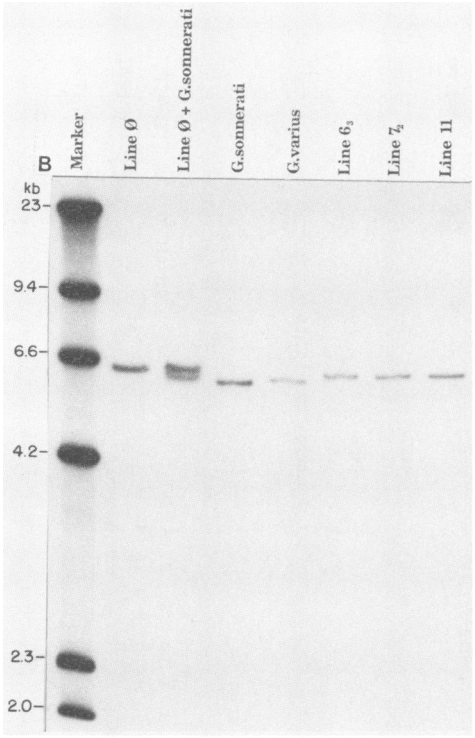


FIG. 6. Solo LTR characterization. (A) Map of the lambda clone and the possible alternative states. R, *Eco*RI; P, *Pst*I; H, *Hin*dIII. (B) Analysis of *Eco*RI-digested DNA from four lines of domestic chicken and two jungle fowl species. Each lane has 2.5 μg of DNA.

reactive sequences in Japanese quail that were more related to EAV-0 than to RSV, and we have cloned an internal *pol-env* fragment representing proviral elements found in quail (6). Sequence analysis reveals slightly more similarity to EAV-0 than RSV, suggesting that EAV-0 elements may represent a family which has entered a *Coturnix-Gallus* progenitor and which has since diverged in sequence. It is not known definitively whether the genomes of other genera contain multiple classes of retroviruses or whether they bear the same proviruses which have since diverged along with the normal host species divergence and which are now only detectable under hybridization at lower stringencies. We intend to test this proposal more rigorously by several approaches, including polymerase chain reaction amplification (36) with EAV-0 LTR-specific primers to test for the presence of the EAV-0 LTR in other genera and cloning studies to isolate EAV-0-related elements from Japanese quail, to address the origin of avian endogenous elements. Molecular cloning and sequencing of positionally conserved, vestigial remnants from the genomes of other genera may identify a putative founding locus and measure the rate of divergence relative to host cellular genes.

Our finding of RAV-0 elements in green jungle fowl was contrary to published findings (6, 14, 15, 53). This presumably indicates horizontal infection by RAV-0 rather than genetic hybridization (cross-breeding) to domestic chickens or red jungle fowl at some point in the recent breeding for the following reasons. First, behavioral and biological breeding barriers argue against interbreeding across species lines (21). Green jungle fowl are behaviorally and phenotypically very different from other jungle fowl as well as geographically isolated from them. F_1 hybrids produced in captivity are generally infertile or at best have very low fertility when backcrossed to *G. gallus* (21). Second, horizontal transmission of avian retroviruses requires close living conditions (34, 40), so that transmission of RAV-0 to *G. varius* is unlikely in the wild, since their ranges rarely overlap. However, a recent infection with RAV-0 while in captivity is very probable. Fibroblasts from red jungle fowl resist infection by RAV-0, the group E virus, due to expression of endogenous viral *env* proteins of the same viral group and are in the viral interference group (C/E), whereas fibroblasts derived from other jungle fowl and Japanese quail support replication of RAV-0 and are designated C/O (52). Therefore, feral *G. sonneratis* and *G. varius* are susceptible to viral infection by all *env* groups of avian leukosis viruses, including RAV-0. Coincidently, the birds from the San Diego zoo were seropositive for REV, although REV sequences are not closely to RSV (22, 23), so REV would not cross-hybridize with RSV or EAV-0.

No domestic chickens other than line-0 have been found to lack RAV-0 elements, and the number of RAV-0 proviruses per individual varies, ranging from 1 to 10, with an average of 4.9 elements (35). Loomis and Gipin (25) and Syvanen (49–51) have suggested that regulation of copy number is perhaps a consequence of a limit to the genetic burden imposed by the acquisition of new germ line elements, though the EAV-0 elements are clearly under less such limitation. Conversely, there is evidence to suggest that there is selective pressure to maintain certain endogenous retroviral elements. Immunoglobulin E-binding factors, lymphokines modulating immunoglobulin E expression which

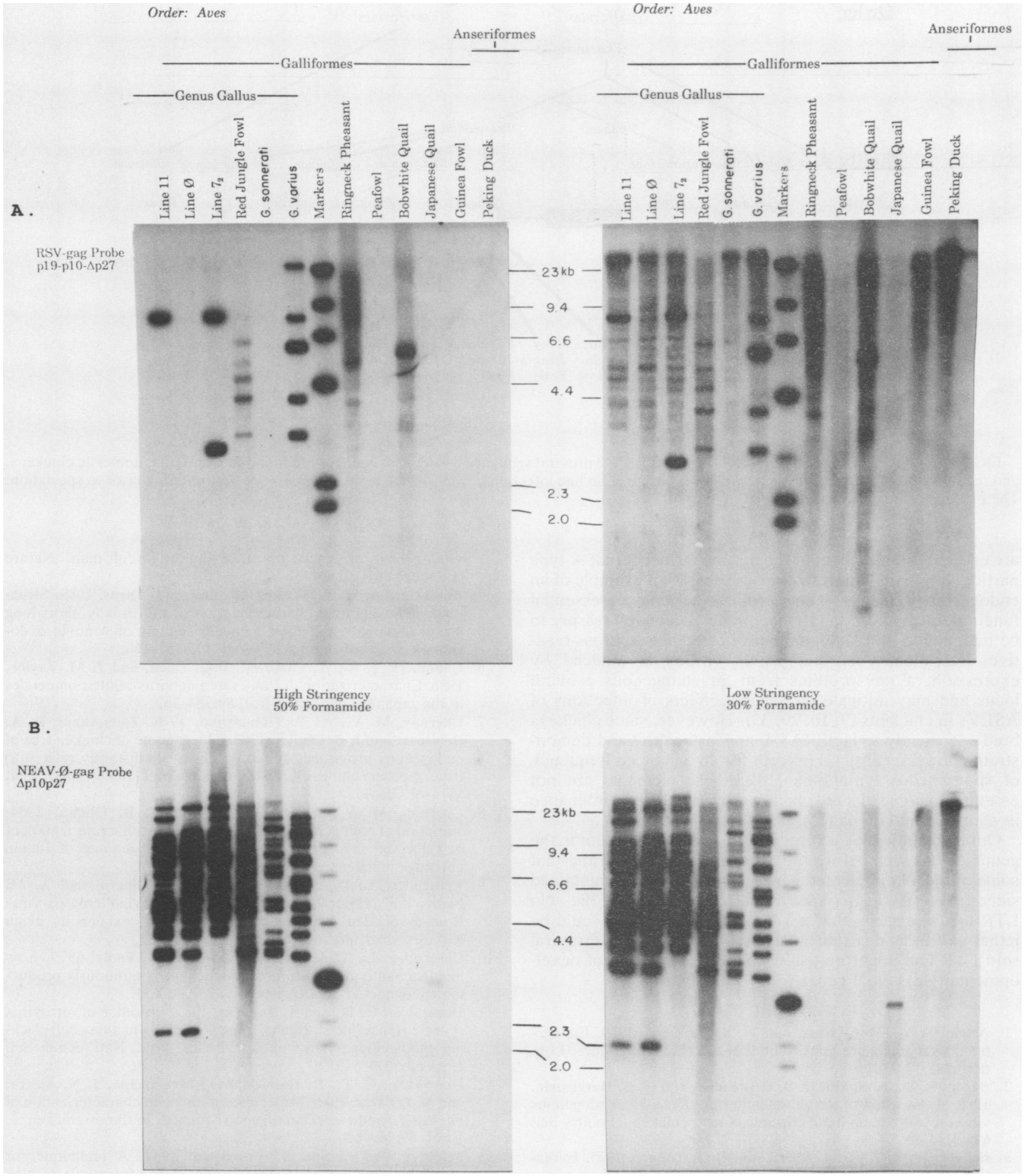


FIG. 7. Detection of RAV-0- and EAV-0-related sequences in avian genera. High- and low-stringency hybridizations of blots of *Eco*RI-digested DNAs (2.5 μg per lane) with the probes described in the legend to Fig. 1. The marker lane includes the conserved 3-kb *Bam*HI-*Eco*RI *gag*-reactive fragment from the EAV-0 clone gp11.

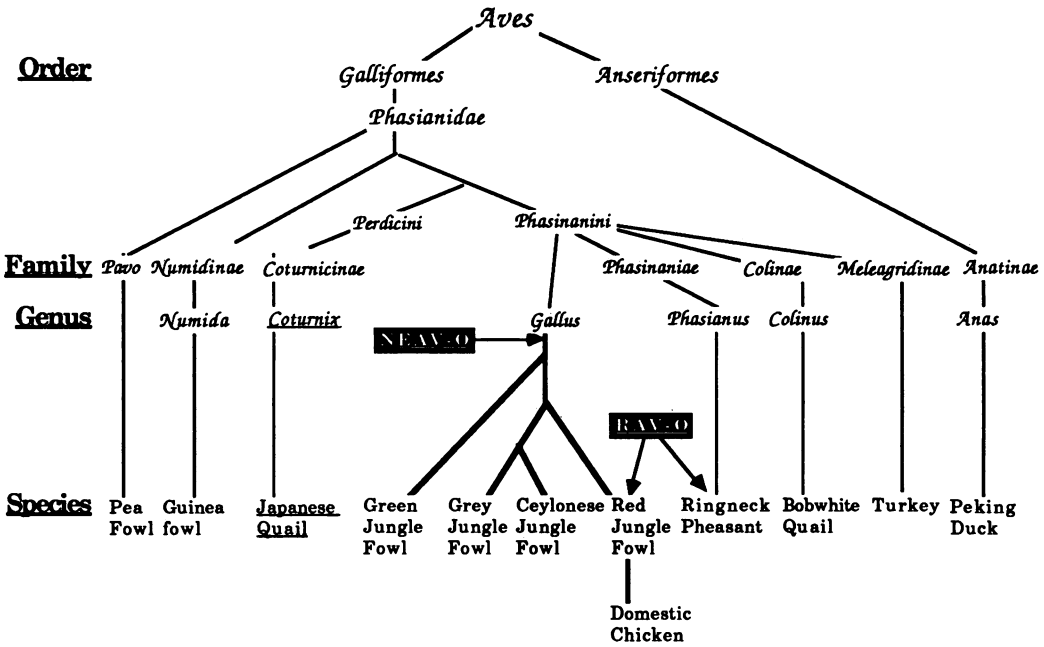


FIG. 8. Phylogenetic distribution of RAV-0 and EAV-0 proviral sequences. RAV-0 sequences are found in pheasants, domestic chickens, and red jungle fowl, supposedly as a result of separate germ line infections. EAV-0 appears in all *Gallus* species, entering prior to speciation. The phylogeny is adapted from Stock et al. (46).

are encoded by members of the murine intracisternal A-type particle gene family of retrotransposons, is an example of an endogenous retroviral gene product serving an essential function for the host and, hence, under selective pressure to be maintained (27). Immunological tolerance to cross-reactive determinants on exogenous virions is induced by expression of *env* proteins from an endogenous proviral locus and may mitigate pathogenic effects of infections of ASLVs in chickens (9, 10, 29, 33). However, since chickens bred specifically to lack RAV-0 appear healthy and demonstrate normal fertility (1) (except for an enhanced frequency of spontaneous lymphoma [10]), these elements are not essential to domesticated chickens despite their maintenance in natural populations (35).

Our data show that several EAV-0 loci were present in the genus *Gallus* well before the acquisition of RAV-0 and that some of the loci presented here have been stably maintained since before *G. gallus* speciation. The analysis of the solo LTR locus suggests that a V^+ state at this locus would be lethal and indicates that analysis of endogenous retroviral solo LTR loci might be useful in characterization of developmentally essential genes in the host species.

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