JOURNAL OF VIROLOGY, Feb. 1995, p. 779–784 Vol. 69, No. 2
0022-538X/95/$04.00+0


# HPRS-103 (Exogenous Avian Leukosis Virus, Subgroup J) Has an *env* Gene Related to Those of Endogenous Elements EAV-0 and E51 and an E Element Found Previously Only in Sarcoma Viruses

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Received 23 August 1994/Accepted 10 November 1994

**The avian leukosis and sarcoma virus (ALSV) group comprises eight subgroups based on envelope properties. HPRS-103, an exogenous retrovirus recently isolated from meat-type chicken lines, is similar to the viruses of these subgroups in group antigen but differs from them in envelope properties and has been assigned to a new subgroup, J. HPRS-103 has a wide host range in birds, and unlike other nontransforming ALSVs which cause late-onset B-cell lymphomas, HPRS-103 causes late-onset myelocytomas. Analysis of the sequence of an infectious clone of the complete proviral genome indicates that HPRS-103 is a multiple recombinant of at least five ALSV sequences and one EAV (endogenous avian retroviral) sequence. The HPRS-103 *env* is most closely related to the *env* gene of the defective EAV-E51 but divergent from those of other ALSV subgroups. Probing of restriction digests of line 0 chicken genomic DNA has identified a novel group of endogenous sequences (EAV-HP) homologous to that of the HPRS-103 *env* gene but different from sequences homologous to EAV and E51. Unlike other replication-competent nontransforming ALSVs, HPRS-103 has an E element in its 3′ noncoding region, as found in many transforming ALSVs. A deletion found in the HPRS-103 U3 EFII enhancer factor-binding site is also found in all replication-defective transforming ALSVs (including MC29, which causes rapid-onset myelocytomas).**

The avian type C retrovirus group includes transformation-competent viruses, such as the avian sarcoma viruses (ASV), and nontransforming viruses, such as the avian leukosis viruses (ALV), as reviewed recently (24). The transformation-competent viruses, which carry oncogenes, induce tumors at a high frequency and with a short incubation period, whereas the transformation-defective viruses, which carry no oncogenes, induce tumors at a low frequency and only after a prolonged incubation period. The nontransforming viruses cause a variety of late-onset neoplasms, with lymphoid leukosis predominating. Until recently, only transformation-competent retroviruses MC29 and CMII (carrying fusions of *gag* with the *myc* oncogene) were known to cause myelocytic leukemia, both with a short incubation period (24). The recent isolation of an exogenous ALV from meat-type chickens, HPRS-103 (25, 28), which causes late-onset myelocytic leukosis (26, 27) is therefore of particular interest, with relevance to the tissue specificity of oncogenesis by retroviruses. HPRS-103 has been shown to represent a previously unknown envelope subgroup, J, as shown by host range and receptor interference assays (25, 28).

We recently isolated a full-length proviral clone of low-passage HPRS-103 from the genomic DNA of line 0 chickens (a strain lacking classical subgroup E endogenous virus loci, i.e., *ev*$^-$). The clone was shown to be infectious upon transfection into line 0 chicken embryo fibroblasts (CEFs). The recovered virus (rHPRS-103) was shown to be of the same subgroup as HPRS-103, and in accord with that fact was the observation that the sequences of the host range determinants of HPRS-103 were very different from those of any of the previously reported subgroups (2). Here, we report the full sequence of HPRS-103 and show that the *env* gene is most closely related to the nonfunctional *env* sequences found recently in ancient endogenous retroviral sequences of the endogenous avian retrovirus (EAV) family (6, 7). Probing the genomes of line 0 (*ev*$^-$) chickens (8) with an HPRS-103 *env*-specific probe under high stringency conditions revealed the presence of about nine homologous endogenous sequences that differ from the sequences which hybridize to EAV-0 or E51 probes. We also show that HPRS-103 is the first natural ALV isolate to carry a copy of the E (or XSR, named for exogenous virus-specific region) element found previously only in sarcoma viruses (33, 38).

## MATERIALS AND METHODS

**Southern blotting of genomic DNA.** Genomic DNA from cultured cells, isolated as described by Sambrook et al. (32), was blotted as described by Southern (36) and hybridized by using a protocol similar to that described by Dunwiddie and Faras (11), prior to autoradiography.

**Subcloning of proviral DNA.** A 19-kb line 0 chicken genomic DNA insert, containing the full-length HPRS-103 provirus, in λGem11 clone recL5 (2) was subcloned into pBluescript II KS(−). Plasmids containing nested deletions of seven individual subclones were derived by using the exonuclease III/S1 nuclease method (13).

**Sequence determination.** Chain termination DNA sequencing was performed with a Sequenase II kit (U.S. Biochemicals) with forward and reverse universal primers. Extension products labelled with α-$^{35}$S-dATP were analyzed by polyacrylamide gel electrophoresis and autoradiography. Gaps in the sequence between adjacent contigs were joined by using specific primers.

**Computer analysis of sequence data.** Sequence data were read with a GrafBar sonic digitizer (Science Accessories Corporation, Stratford, Conn.) and assem-

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bled by using the programs of Staden (37). Database searches were performed with FASTA, and sequences were analyzed with programs of the GCG package (Sequence Analysis Software Package 7.2; Genetics Computer Group, Madison, Wis.) (9), DNA Strider 1.2 (19), and CAD Gene 1.05 (Genetic Technology Corporation).

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number Z46390.

## RESULTS

**Sequence of the full-length proviral clone of HPRS-103.** The isolation and characterization of a full-length infectious DNA clone (recL5, in λGem11) of HPRS-103 (which had undergone minimal passage in CEFs from the original isolate) from infected CEF genomic DNA, as well as that of a partial clone (recL7), will be described elsewhere (2). The full genomic proviral sequence of HPRS-103 is 7,841 nucleotides long with a genetic organization typical of replication-competent type C retroviruses (i.e., long terminal repeat (LTR)-leader-*gag*/*pol*-*env*-LTR).

**The *gag* and *pol* genes of HPRS-103 are well conserved.** The *gag* and *pol* genes of HPRS-103 (bases 604 to 2706 and 2727 to 5345, respectively) are well conserved with those of other avian leukosis and sarcoma viruses (ALSVs) (showing 96 to 97% overall identity to the genes of subgroups A, D, and C).

A major difference between the *pol* gene of HPRS-103 and those of Prague C (Pr-C) Rous-associated virus-1 (RAV-1) and subgroup D is that there is a C-to-T change found in both proviral clones of HPRS-103 at base 5346, forming a stop codon and resulting in the Pol of HPRS-103 being 22 amino acids shorter.

**The *env* gene of HPRS-103 is highly divergent.** As reported elsewhere (2), the sequence encoding the SU (gp85) domain of HPRS-103 (bases 5476 to 6399) has only 40% identity overall to the corresponding sequences of other subgroups (in comparison, the SU-encoding sequences of other subgroups show 80 to 85% identity to each other). The N- and C-terminal amino acid sequences of HPRS-103 SU are better conserved than the middle sequences (residues 54 to 253), which differ significantly from those of other subgroups, in conserved as well as in variable regions (most of the variations among other subgroups occur in variable regions) (5, 10).

Sequences encoding the TM (gp37) domain of HPRS-103 (bases 6400 to 6990) show 65% overall nucleotide identity to the sequences of other subgroups (which show 92 to 95% identity to each other).

**The *env* gene sequence of HPRS-103 is most closely related to the defective *env* sequence of E51, a member of the EAV sequence family.** Database searches revealed that the *env* gene of HPRS-103 was most similar (75% identity) to the defective *env* gene of E51, a member of the EAV family of endogenous retroviral elements found initially in line 0 $ev^-$ chickens (6). In contrast, it was reported that the E51 SU-coding sequence has an overall identity of less than 50% compared with the corresponding sequence of the subgroup C viruses (6). The SU-coding sequence of E51 is defective, disrupted by five separate frameshifts. Allowing for the frameshifts, the overall homology between a corrected SU of E51 and that of HPRS-103 would be 66% amino acid identity (for comparison, the homology between the corrected SU of E51 and those of ALVs of subgroups A to D would be 28 to 32%). Thus, the SU of HPRS-103 is clearly similar to the defective SU of E51 and divergent from those of other ALVs (Fig. 1).

**The transmembrane region of HPRS-103 TM is encoded by a unique insertion.** The transmembrane region of the TM of HPRS-103 is encoded by an insertion (bases 6796 to 7015) relative to RAV-1. Moreover, the 3′ end of the subgroup A TM-coding sequence most closely matches a region downstream of the insertion (and therefore downstream of the HPRS-103 TM-coding sequence).

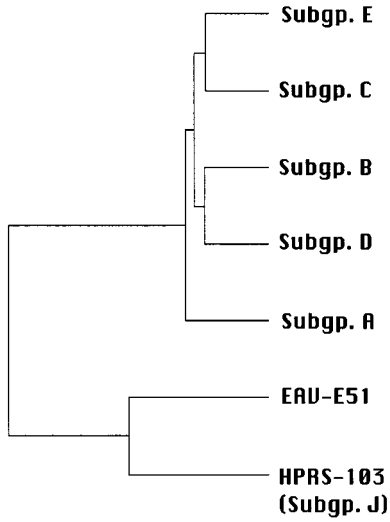


FIG. 1. Relationship of SU-encoding sequences. Sequences encoding the SU domains of HPRS-103, EAV-E51, and viruses of subgroups A to E were aligned by using the Genetics Computer Group program PILEUP, producing a dendrogram which was plotted with the FIGURE program. The sequences used for comparison are listed in the databases under the following names and accession numbers (the subgroup, where appropriate, and the nature of the sequence are also indicated). RAV-1 (subgroup A): ALVCG, M37980, complete genome (3); SR-RSV-D (subgroup D): ALRSRD, D10652, complete genome (16); RSV Pr-C (subgroup C): RERSV6, V01197, complete genome (33); EAV-E51: GGTRANSA, M95189, endogenous *env*-LTR (6); RAV-0 (subgroup E): REALRG02, M12172, gp85 (10); RAV-2 (subgroup B): RERAVENV, K02374, *env*-LTR-*gag* (4).

The hydrophobicity profile of sequences encoded by the insertion suggests that it encodes a functional transmembrane domain for the HPRS-103 TM even though it shows only 23% amino acid identity to the transmembrane domain of other subgroups and 45% identity to that of E51.

Whereas the TM sequence upstream of the insertion in HPRS-103 shows about 65% identity to those of both EAV-E51 and the ALSVs, the sequence immediately downstream of the insertion shows 97% identity to those of the ALSVs but only 41% identity to that of E51. This suggests that the now redundant and nonfunctional downstream transmembrane sequence is of ALSV origin.

**Location of the boundaries of the *env* gene recombination event.** The 3′ recombination boundary in the HPRS-103 sequence is likely to be between the insertion and the redundant transmembrane sequence (after nucleotide 7015). A sequence upstream of this point is most closely related to that of EAV-E51, but downstream it is nearly identical to those of other ALSV subgroups (Fig. 2). A short stretch of homology between HPRS-103, EAV-E51, and the ALSVs, which could have promoted the aberrant recombination event, giving rise to two transmembrane region-encoding sequences in HPRS-103, was found at the boundary (Fig. 2B).

Upstream of the position of the *pol* stop codon of other subgroups (equivalent to bases 5412 to 5414 of HPRS-103), the HPRS-103 genome is well conserved with those of the other ALVs but not with that of EAV-E51. Between the ALSV *pol* stop codon and the site for *env* signal peptide cleavage (after 5475), HPRS-103 shows only low identity to both EAV-E51 and the ALSVs. Downstream of the signal cleavage site,

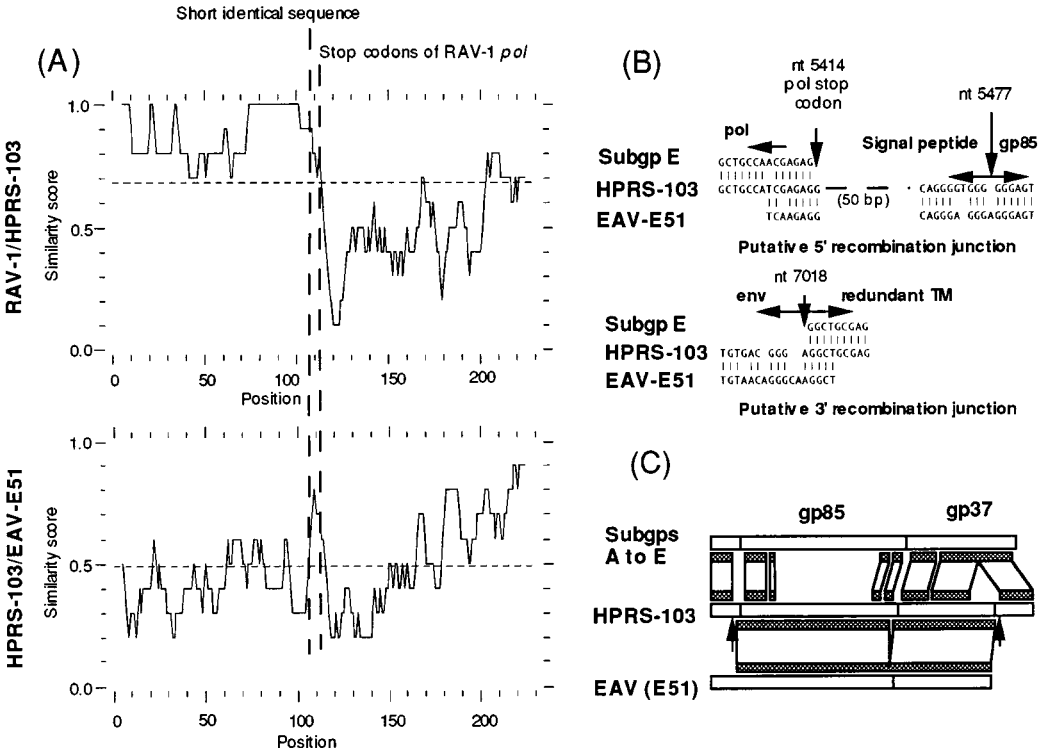


FIG. 2. Recombination junctions in the *env* gene of HPRS-103 between a virus of subgroup A to E and an EAV-like sequence. (A) Homology between *env* leader sequences of HPRS-103, RAV-1, and EAV-E51 depicted by using the Genetics Computer Group PLOTSIMILARITY program from multiple sequence alignments generated with the PILEUP program. Horizontal dashed lines show the average similarity calculated for each pair of sequences. The upper plot shows the homology between HPRS-103 and RAV-1. The lower plot shows the homology between HPRS-103 and EAV-E51. There is a short region of a higher sequence identity between HPRS-103 and EAV-E51, corresponding to sequences in RAV-1 around the *pol* stop codons (this region and the stop codons are indicated by vertical dashed lines), followed by a short region of lower sequence identity and thereafter, an increased sequence identity downstream of this region in the SU-coding domain (see panel C). (B) Short homology overlap sequences at the putative 5′ and 3′ recombination junctions. The position of the *pol* stop codon of RAV-1 is indicated by a vertical arrow as are the positions of the boundaries between the signal peptide and gp85 and between *env* and the redundant TM. nt, nucleotide. (C) Homology between the *env* genes of HPRS-103, viruses of ALSV subgroups A to E, and EAV-E51. The diagram is based on multiple sequence alignments of *env* genes (sources of sequences were as described in Materials and Methods). Highlighted regions show more than 50% homology (nucleic acid identity). Arrows indicate the positions in the *env* of HPRS-103 of the two short homologous regions shown in panel B.

HPRS-103 is closely related to EAV-E51 (Fig. 2C). There is a clear break in homology between HPRS-103 and subgroup A, initiated by an insert which consists of a $(CTT)_3$ repeat immediately after the ALSV *pol* stop codon (Fig. 2A). It is probable that this break in homology between HPRS-103 and subgroup A coincides with the 5′ recombination site. There is no corresponding sharp transition to higher homology with E51; instead, homology increases steadily over a range of about 100 bp after the breakpoint (Fig. 2A).

**HPRS-103 *env* gene homologs are present in the genome of line 0 chickens.** The low homology of HPRS-103 SU to that of RAV-1, combined with its high homology to that of EAV-E51, suggested that the HPRS-103 SU-coding domain might be derived from endogenous sequences rather than from exogenous virus. To investigate this possibility, restriction digests of HPRS-103-infected and uninfected line 0 CEF DNA were analyzed by Southern blotting, at high stringency, with HPRS-103 full-length *env* (located between bases 5219 and 7300) or a probe from the middle of the SU-coding sequence (located between bases 5603 and 6211). Under the conditions used, the HPRS-103 SU probe would not be expected to hybridize to the cellular E51 sequence.

Southern blots for HPRS-103-infected and uninfected line 0 CEF DNAs showed identical restriction enzyme digestion profiles, with both the full-length *env* and the SU middle-region probes (at high stringency), except for those bands that represent HPRS-103 proviral forms (4- to 4.6-kb fragments for *Bam*HI, 5.5- and 7.8-kb fragments for *Eco*RI, and a 4.5-kb fragment for *Pst*I) (Fig. 3, lanes I). Digestion of line 0 CEF DNA with *Eco*RI and *Pst*I produced 10 and 11 hybridizing DNA fragments, respectively, with sizes of 4.6 to 23 kb and 4.5 to 19 kb, respectively. Restriction profiles identified with HPRS-103 SU probes in the line 0 chicken genome are different from the profiles identified with EAV-0 and EAV-E51 probes in the same chicken line, both in the position and in the number of hybridizing bands (6).

Control RAV-1 *env*-specific DNA fragments hybridized only to the full-length HPRS-103 *env* (Fig. 3A, lanes RAV-1) but not to the SU probe (Fig. 3B, lanes RAV-1). A 4.6-kb DNA band which hybridized to the HPRS-103 SU probe is actually the plasmid vector fragment from pRCAS and not the RAV-1 *env* DNA fragment. A corresponding faint band (Fig. 3A) for RAV-1 DNA was also seen with the full-length *env* probe.

**The 3′ noncoding sequence contains an E (or XSR) element.** Downstream of the redundant transmembrane sequence, the 3′ noncoding region of HPRS-103 contains a single copy of the direct repeat (DR1) element, also found as a single copy in ALVs but as two copies flanking the *src* gene in ASVs. Downstream of DR1 is a copy of the E (or XSR) element, found in ASVs in which a single copy is present on either side of the *src* gene (upstream of *src* in the Schmidt-Ruppin [SR-A] strain of Rous sarcoma virus [RSV] and downstream in the Pr-C strain)

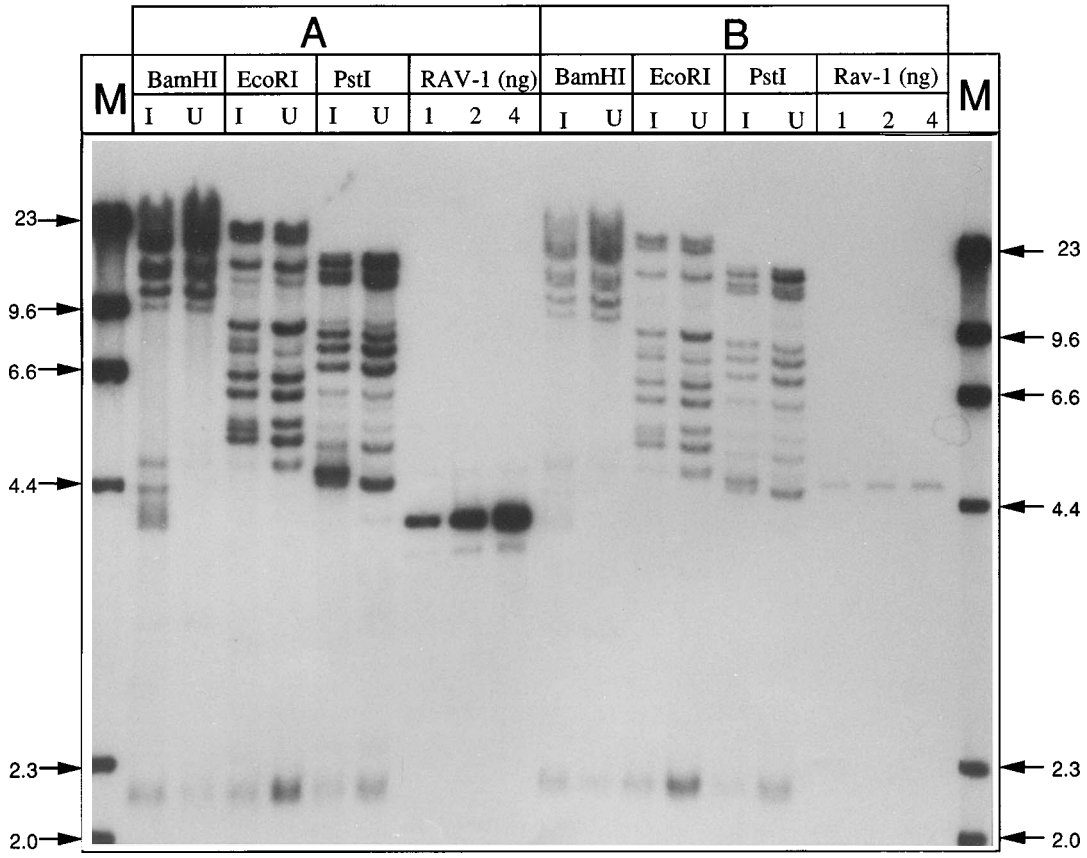


FIG. 3. Characterization of HPRS-103 *env*-related endogenous DNA sequences in the line 0 chicken genome. HPRS-103 infected (I) and uninfected (U) CEF DNAs were digested with restriction enzymes. Digests were analyzed by Southern blot hybridization with the full-length *env* (A) or the central region (B) of the HPRS-103 SU-coding sequence. *Bam*HI-digested pRCAS was used as a control (RAV-1). RAV-1 DNA hybridized only to the full-length *env* probe of HPRS-103 (A). In panel B, the hybridizing bands are plasmid DNA fragments that do not contain any ALV *env* DNA sequences. Size markers (lanes M) are in kilobase pairs.

(4). The E sequence of HPRS-103 is well conserved with that of RSV Pr-C, except that there is a 6-base deletion upstream of the PU 2 site (C/EBP binding) (31) in HPRS-103 (Fig. 4A).

**LTRs.** Two copies of the LTR (325 bp long) are separately located at each end of the proviral genome. The U3 element is 224 bp long, the R element is 21 bp long (bases 225 to 245), and the U5 region is 80 bp long (bases 246 to 325). The R and U5 regions of HPRS-103 are well conserved with other subgroups and are most closely related to the corresponding regions of ASVs Y73 (17, 20) and UR2 (23), respectively. Indeed, Y73 (and also CT10 [20]) has a sequence identical to that of HPRS-103 for the R region.

Overall, the U3 element of HPRS-103 is more closely related (92% nucleic acid identity) to those of Y73 and CT10, as well as to that of subgroup B (90% identity), than to the U3 elements of other ALSVs. Whereas RSV and ALVs of subgroups A to D possess IR 2 (which forms part of the EFII binding site [34], a major enhancer element for ALSVs), this element is deleted from HPRS-103, Y73, CT10, MC29 (29), and avian erythroblastosis virus (39). Indeed, Y73, CT10, and MC29 have the same 19-bp deletion as HPRS-103 (Fig. 4B); avian erythroblastosis virus has a smaller deletion. RAV-0 (14) and Fujinama sarcoma virus (35), which are closely related to each other, are highly divergent in U3 from the other viruses. The U3 element of CT10, which in structure and overall homology is very similar to that of HPRS-103, differs considerably within IR1 (differing at 8 of 24 nucleotides). The U3 element of HPRS-103 is very similar to that of Y73 (except that Y73 has an 11-base deletion after base 109 of U3), especially at the 5′ end. HPRS-103 is, therefore, most closely related to both Y73 and CT10 in the R region and to Y73 and CT10 at the 5′ and 3′ ends of U3, respectively.

**The nontranslated leader sequence.** Five ATG codons exist in the HPRS-103 leader (which is most closely related to those of UR2 and Y73) at bases 265, 306, 315, 337, and 423. The sixth ATG at nucleotide 604, conserved in all ALSV subgroups, introduces a large open reading frame, encoding Gag (3, 4, 16, 33). The first, second, and fifth ATGs are conserved in all avian retroviruses; each introduces a short reading frame of less than 50 bp. The third ATG and the fourth, located in the conserved primer binding site (33), are unique to HPRS-103. So far, little is known about the functions of these multiple upstream ATGs (12), though they do seem to be implicated in virus replication (21, 22).

## DISCUSSION

Most ALVs and ASVs studied in the laboratory originate from isolates made many years ago, mainly from laying-type birds. The full-length sequence we report here is of an ALV only recently isolated from meat-type birds. The virus is novel in terms of its envelope properties and its wide host range among chicken lines and because of the fact that it alone

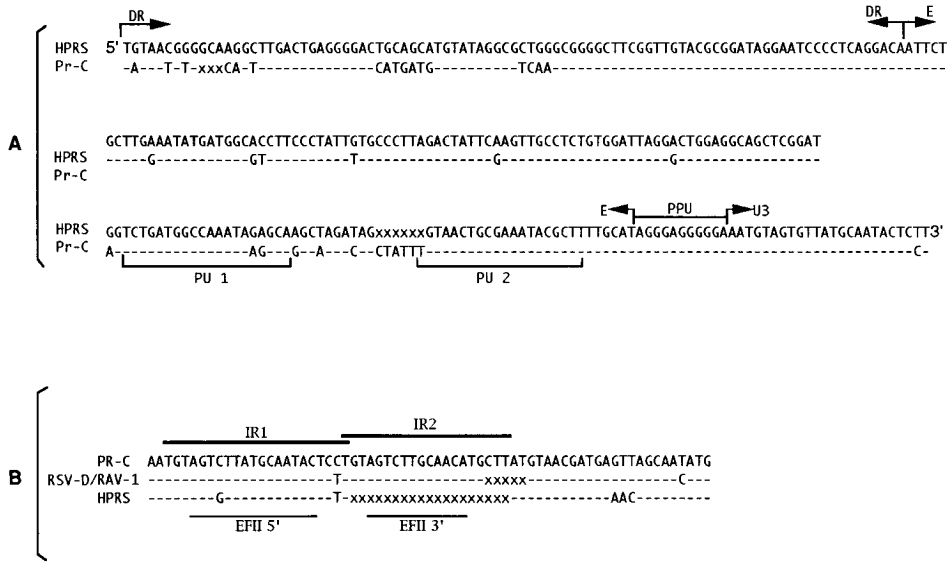


FIG. 4. Sequences of ALSV 3′ noncoding regions and LTRs showing potential enhancer factor-binding sites. (A) The 3′ noncoding region and C/EBP-binding sites, PU 1 and PU 2 (31). (B) The 5′ terminus of the ALSV LTR U3 showing C/EBP-like factor-binding sites (30, 31, 34). Sources of sequences were as described in the legend to Fig. 1. PPU, polypurine tract; IR, imperfect repeat; DR, direct repeat.

among nontransforming ALVs causes late-onset myelocytomas, instead of the usual B-cell lymphomas.

Even though HPRS-103 was assigned to a different envelope subgroup (25, 28), the extent of the sequence divergence in the host range determinants was surprising (2). Even more surprising was the subsequent discovery that the sequence encompassing the host range determinants was more closely related to the endogenous EAV sequence E51 (Fig. 1).

Most EAV-0 proviruses are deleted in *env* (6, 7), but a related sequence, E51, had a full-length *env* gene, defective in SU (6). High-stringency Southern blot hybridization showed that there are many proviral elements in the chicken genome related to E51, and the conservation of these elements indicated that they may be even more ancient than EAV-0 (6).

While it is clear that the *env* gene of HPRS-103 is more closely related to that of EAV element E51 than to the *env* gene of classical ALVs, its relationship to EAV-0 is a little more enigmatic because of the reported *env* defectiveness of the EAV-0 clones. To the 5′ side of the EAV-0 *env* deletions, HPRS-103 is more closely related to E51 (59% nucleic acid identity) than to EAV-0 (52%), and it is more closely related to E51 than E51 is to EAV-0 (54%). To the 3′ side of the EAV-0 *env* deletions, HPRS-103 is slightly more closely related to E51 (70% nucleic acid identity) than to EAV-0 (69%), but it is more distantly related to E51 than E51 is to EAV-0 (76%). Thus, the *env* gene of HPRS-103 either is derived from an EAV-like sequence different from any EAV sequence yet reported (whether EAV-0- or E51-like) or has diverged considerably from its ancestral element, which could have been more closely related to either EAV-0 or E51.

Irrespective of the identity of the actual ancestral sequence, HPRS-103 can be regarded as an example, previously undescribed, of an exogenous counterpart of EAV. It is not clear whether HPRS-103 acquired the *env* gene by recombination with another such exogenous counterpart of EAV or with endogenous sequences. While the EAV-E51 *env* sequence is defective and somewhat divergent from that of HPRS-103, it is possible that other such sequences encode, or once encoded, functional *env* genes. Indeed, our results show that *ev*$^-$ line 0 chickens carry a number of sequences more closely related to the *env* gene of HPRS-103 than to the defective *env* genes of EAV-0 or E51. It remains to be determined whether any of those sequences, which we propose to call EAV-HP, encode functional *env*.

The discovery of an E element in the 3′ noncoding region was also surprising. This element, also known as F2 (4, 18) and XSR (38), had only been found in replication-competent RSVs (such as RSV Pr-C, RSV SR-A, and SR-RSV-D) but not in naturally occurring, replication-competent ALVs such as RAV-1, RAV-2, and RAV-0 (3, 4). A recombinant (NTRE-7) selected from cells infected in vitro with transformation-defective RSV Pr-B B and RAV-0 did inherit E from the RSV parent (38). The E element is found 5′ of v-*src* in the genome of RSV SR but 3′ of v-*src* in RSV Pr (33).

The discovery of a truncated Pol in HPRS-103 was unexpected, given the strong amino acid sequence conservation in other ALSVs. It has been shown that cleavage of Pol, to generate the C terminus of IN (pp32), occurs at a position 15 amino acid residues upstream of the truncation (1) and that the small, cleaved C-terminal domain was unnecessary for viral replication in vitro (15). The truncation in the Pol of HPRS-103 is, therefore, unlikely to affect severely the phenotype of HPRS-103.

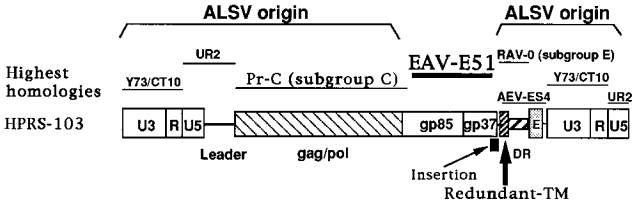


FIG. 5. Schematic showing the overall sequence relationship of HPRS-103 to the ALSVs and EAV-E51. The boxed sequence line shows the structure of the HPRS-103 proviral genome. The short lines above indicate the viral sequences which are most closely related to those parts of the HPRS-103 genome. The position of the insertion is also indicated.

It is clear from sequence comparisons that, apart from the acquisition of the EAV-like *env* sequences, HPRS-103 is more closely related to several other ALSVs in different parts of the genome (summarized in Fig. 5). HPRS-103 is, therefore, separated from the other ALSVs by a number of recombination events, though it is not clear in which lineage(s) those events have taken place. It is intriguing to speculate that the isolation of HPRS-103 from meat-type birds might account for some of its peculiar features, raising the possibility that other such isolates might share at least some of those features.

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