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Novel endogenous retroviral sequences in the chicken genome closely related to HPRS-103 (subgroup J) avian leukosis virus

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HPRS-103, the prototype of avian leukosis virus (ALV) subgroup J, is a recently identified retrovirus associated with myeloid leukosis in meat-type chickens. Although this virus shows high sequence identity to other ALV subgroups within the *gag* and *pol* genes, its *env* gene is highly diverged (with only about 40% sequence identity) from other ALV subgroups. On the other hand, the sequence of the *env* gene of HPRS-103 was 75% identical to that of E51, a member of the EAV family of endogenous avian retroviruses. It is reported here that the chicken genome also contains another EAV-related element, EAV-HP, showing much greater sequence identity (over 97%) to the HPRS-103 *env* gene. Southern blotting analysis showed that EAV-HP-related sequences were distinct from EAV-0 and were present in all lines of chicken examined and in grey jungle fowl, but were absent from several other avian species. The potential role of these endogenous sequences in the evolution of ALV subgroup J viruses is discussed.

Introduction

Avian species have co-existed with retroviruses for millions of years, during which time repeated virus colonization of the germline has resulted in chromosomally integrated endogenous retroviruses (ERVs) that are inherited in a Mendelian fashion. The avian genome contains many families of ERVs such as *ALVE* (*ev* loci), CR1, EAV and ART-CH (reviewed by Crittenden, 1991). Of these, the *ALVE* family is perhaps the best studied (Rovigatti & Astrin, 1983). Most lines of chicken carry up to eight copies of these elements at different genomic locations. About 22 such elements have been documented in White Leghorns and probably many more exist in other heavy breeds (Crittenden & Salter, 1998). While the expression of some of these loci results in the production of entirely defective proviruses, expression of other loci (e.g. *ALVE*2, expressing the prototype endogenous RAV-0 virus) can produce a complete genome (Hughes *et al.*, 1981). The *ALVE* family of ERVs has been shown to be not essential for birds, as it was possible to breed a chicken line (line 0) free from these elements (Astrin *et al.*, 1979). It was shown subsequently that the line 0 chicken genome contained several other independent and unique ERVs. One of these, designated the EAV-0 family (Boyce-Jacino *et al.*, 1989), comprised an extensive group of retrovirus elements, most of which showed unique deletions within different parts of the genome including the *env* gene. Some members of this family, such as E51, had potentially full-length *env* genes, but these were defective because of stop codons and multiple frame shifts (Boyce-Jacino *et al.*, 1992). These studies also showed that EAV-0 elements did not account for all the avian leukosis virus (ALV) *env*-related sequences in line 0 birds, suggesting that other elements distantly related to EAV-0 were present in avian DNA.

Unlike mammalian ERVs, the avian endogenous elements were not known to have any closely related exogenous counterpart until the new J subgroup ALV, HPRS-103, was isolated (Payne *et al.*, 1991). The sequence of the *env* gene of HPRS-103 is over 75% identical to that of the *env* gene of the E51 element (Bai *et al.*, 1995). However, as the two sequences are not identical and E51 is a defective provirus, it is possible that HPRS-103 virus acquired its *env* sequences from other EAV-0-related endogenous elements encoding more closely related functional genes. In support of this, we have observed ERV elements in genomic DNA that are closely related to HPRS-103, by probing with surface domain (SU)-specific probes of the *env* gene. These elements were suggested to belong to a new subfamily of ERV elements designated EAV-HP (Bai *et al.*, 1995). However, as the probes used in these

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The sequence of the EAV-HP *env* region has been deposited in GenBank as accession no. AJ222690.

experiments included regions with high degrees of identity to other members of the EAV-0 family, such as E51 (Boyce-Jacino *et al.*, 1992), it was unclear whether the EAV-HP sequences were distinct and represented a new subfamily of ERVs. In order to confirm that EAV-HP elements represent a new subfamily of EAV elements showing close relationship to ALV subgroup J, we have sequenced part of the *env* region of these elements from meat-type chickens. The sequence of EAV-HP is distinct from E51, with an intact coding region showing high sequence identity to the envelope glycoprotein of HPRS-103, supporting the notion that ALV subgroup J evolved by recombination with these sequences. We also show that these sequences are present in all lines of chicken we have examined and show a distribution very similar to the EAV-0 family of ERVs.

Methods

■ **Preparation of genomic DNA and Southern blotting.** Genomic DNA was prepared from embryos or embryo fibroblast cultures (Payne *et al.*, 1992) of the following avian species: chicken (*Gallus gallus domesticus*) (lines 15I, C, N, 0, 6_1, Light Sussex, 7_2 and Sykes Rhode Island Red), ring-necked pheasant (*Phasianus colchicus*), Guinea fowl (*Numida meleagris*), turkey (*Meleagris gallopavo*), Muscovy duck (*Cairina moschata*), domestic duck (*Anas platyrhynchos*), Japanese quail (*Coturnix japonica*) and grey jungle fowl (*G. sonneratii*). The following cell lines were used as sources of mammalian genomic DNA: L929 (mouse), a human osteosarcoma cell line, MDBK (cow) and Vero (African green monkey). DNA was isolated with a lysis solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate and 1% Sarkosyl, followed by phenol–chloroform extraction and ethanol precipitation (Smith *et al.*, 1998). DNA was also extracted from yeast artificial chromosome (YAC) clones obtained by screening the Compton/ICRF YAC chicken library (Toye *et al.*, 1997) with an EAV-HP *env*-specific probe (see below). DNA was digested, restriction fragments were resolved by electrophoresis through 1% agarose gels and transferred to nylon membranes by standard methods (Sambrook *et al.*, 1989).

■ **Oligonucleotide primers.** The following primers (5′ to 3′) were used for PCR amplification. They were designed according to published HPRS-103 sequences (Bai *et al.*, 1995) and their relative positions are indicated on the schematic map (Fig. 1). H2 (nt 5958–5981) ACTGGTGAATCCACAATATCTACG; H3 (nt 5659–5679) AACAACACCGATTTAGCCAGC; H4 (nt 6188–6207) CAACACCTCTGGCTGTTCCC; H5 (nt 5258–5277) GGATGAGGTGACTAAGAAAG; H7 (nt 5783–5801) CGAACCAAAGGTAACACACG; H8 (nt 5956–5979) TGGTGAATCCACAATATCTACGAC; 37.1 (nt 6983–7002) TCGGAACCTACAGCTGCTCC; AJ1 (nt 7039–7062) ATGAACGGCCCATTC(C/T)CCTATTCC.

■ **Labelled probes and hybridization.** A plasmid clone encoding the *env*–long terminal repeat (LTR) region of EAV-0 (Benkel & Gavora, 1993) was kindly provided by B. F. Benkel (Centre for Food and Animal Research, Agriculture Canada, Ottawa, Canada). EAV-HP *env*-specific probes were prepared from the 548 bp PCR product obtained with primers H3 and H4. [α-^{32}P]dCTP-labelled DNA probes were derived

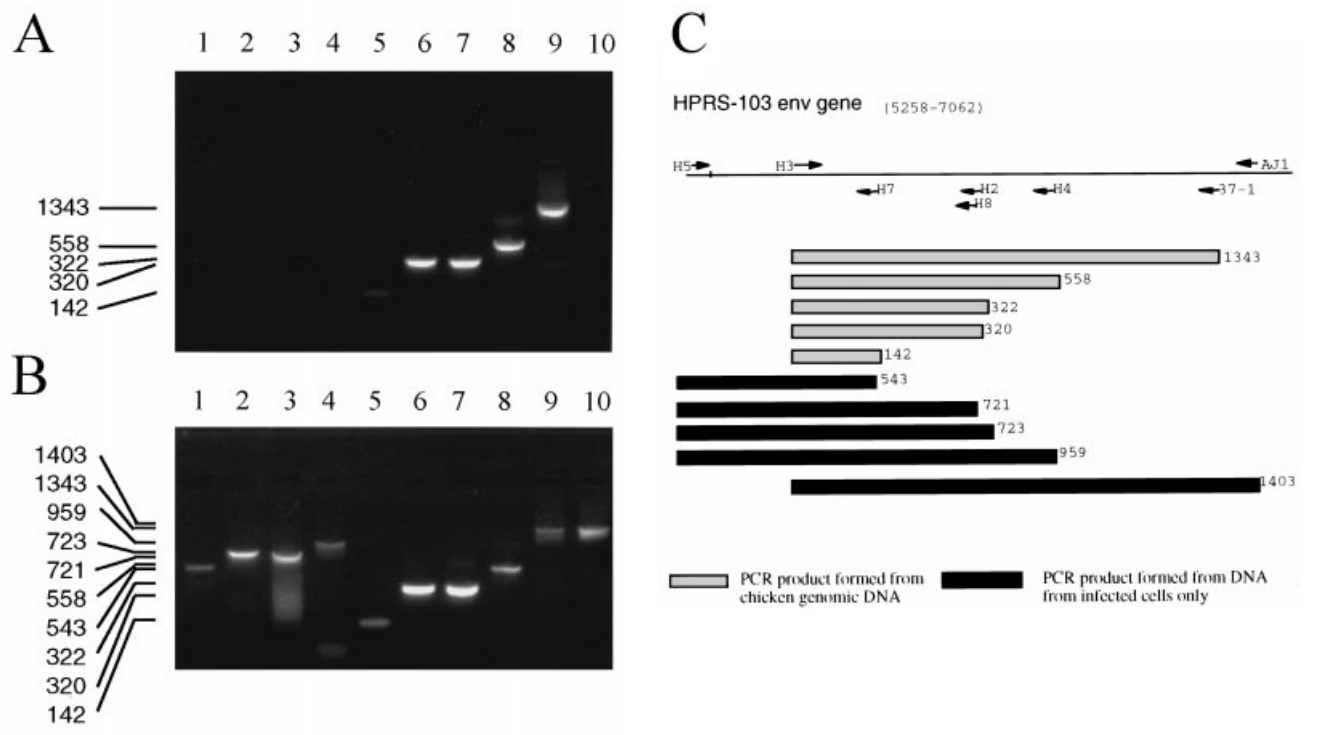

Fig. 1. PCR amplification of EAV-HP and HPRS-103 sequences using different pairs of primers. (A) PCR products obtained with uninfected line 0 DNA as template. (B) PCR products obtained with HPRS-103-infected line 0 DNA as the template. Primer pairs used were: lanes 1, H5–H7; 2, H5–H8; 3, H5–H2; 4, H5–H4; 5, H3–H7; 6, H3–H8; 7, H3–H2; 8, H3–H4; 9, H3–37.1; 10, H3–AJ1. (C) Diagram showing the positions of the primers and the expected sizes of the products.

from gel-purified PCR products or plasmid DNA using the RTS RadPrime labelling kit (Gibco BRL). Hybridization was carried out under stringent (50% formamide) conditions (Dunwiddie & Faras, 1985).

■ **PCR, cloning and nucleotide sequence analysis.** PCR amplification of the EAV-HP sequences was performed in a total volume of 100 µl with 300 ng each primer, 0·5–1 µg target genomic DNA and 0·2 mM each dNTP in a buffer containing 2 mM $MgCl_2$, 100 mM Tris–HCl (pH 8·3), 500 mM KCl, 0·1% gelatin and 2·5 U *Taq* DNA polymerase (Promega). Reaction conditions were 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min for 30 cycles followed by extension at 72 °C for 10 min. The 1·3 kbp PCR product obtained with primers H3 and 37.1 from line 21 chicken was gel-purified and cloned into pGEM-T vector (Promega), following protocols supplied by the manufacturer. Sequences were determined with vector-specific and internal oligonucleotide primers by using the ABI PRISM dye terminator cycle sequencing kit with AmpliTaq DNA polymerase FS (Perkin-Elmer) on an ABI 373A DNA sequencer. Sequence analysis was performed using the Genetics Computer Group software.

Results

Amplification of the *env* region of EAV-HP by PCR

The presence of HPRS-103 *env*-related EAV-HP sequences in the chicken genome was initially identified when genomic DNA from uninfected line 0 chicken was probed with HPRS-103 SU-specific probes (Bai *et al*., 1995). Subsequent studies using PCR with primers H3 and H4 with this DNA gave a 548 bp product identical to that obtained from DNA from HPRS-103-infected cells (Fig. 1). The sequence of this PCR product showed almost 60% identity to E51 and over 97% to the HPRS-103 *env* gene (data not shown). In order to assess the sequence differences and the collinearity between the *env* genes of HPRS-103 and EAV-HP elements, a range of PCR amplifications was carried out with sets of primers derived from the HPRS-103 *env* gene sequence, with uninfected and infected DNA from line 0 birds as the template. Products of identical sizes were obtained from both DNA templates with primer pairs H3–H7, H3–H8, H3–H2, H3–H4 and H3–37.1 (Fig. 1). However when the primer H3 was replaced by a more upstream primer, H5, or when the primer 37.1 was replaced by more downstream primer, AJ1, PCR products were observed only with DNA from HPRS-103-infected cells (Fig. 1).

Distribution of EAV-HP *env*-related sequences in the avian genome

In order to establish that EAV-0 and EAV-HP elements in the genome represented distinct sequences, Southern blots of line N and 15I genomic DNA, digested with different restriction enzymes, were probed with EAV-HP and EAV-0 labelled probes. The distinct hybridization patterns obtained with these probes suggested that the sequences, although closely related, were not identical (Fig. 2). The differences in the number of bands obtained with different restriction enzymes on the two blots also suggested that EAV-HP proviruses were less abundant than the 50 proviral copies of EAV-0 observed in the chicken genome (Boyce-Jacino *et al*., 1992).

Having shown that the EAV-HP elements were distinct from EAV-0, we wanted to examine whether the conservation of EAV-HP elements among different species of bird and lines of chicken was similar to that of EAV-0. Southern blots of *Eco*RI-digested genomic DNA from eight lines of chicken as well as several from other avian and mammalian species were hybridized under high stringency with EAV-HP *env*-specific probes. Complex hybridization patterns, reflecting multiple copies of proviruses, were seen in all lines of chicken (Fig. 3). Single DNA samples obtained from line 0 chicken and *G. sonneratii* also gave positive signals with the EAV-HP *env*-specific probe. No hybridization signals were seen in blots of the DNA of other avian species such as ring-necked pheasant, Guinea fowl, turkey, Muscovy duck, domestic duck and Japanese quail or mammalian species such as human, mouse, cow or African green monkey (Vero cells).

Identification of EAV-HP-containing YACs

Screening of a chicken line N genomic YAC library with the H3–H4 PCR product identified 30 positive clones; five of these clones were selected for subsequent analysis. Southern blotting of YAC DNA from these five clones digested with different restriction enzymes showed differing fragment patterns except in the case of YAC clones C and D, which produced virtually identical fragment patterns and presumably contained the same endogenous locus (Fig. 4).

Chromosomal mapping of EAV-HP

Mapping studies were carried out using the Compton mapping reference population, which was derived from a cross between the inbred White Leghorn lines N and 15I (Bumstead & Palyga, 1992). Southern blots of *Hin*dIII- or *Eco*RI-digested DNA of the parent birds and 56 progeny of this population were probed with the H3–H4 PCR product. Fifteen *Eco*RI and 12 *Hin*dIII fragments were observed, rather less than the number of EAV-0 bands reported for this population, which was approximately 30. The sizes of the EAV-HP fragments also differed from those of the EAV-0 fragments, suggesting that there was no overlap between these loci. Ten EAV-HP fragments segregated in the progeny of this cross and the segregation patterns of these fragments identified six distinct EAV-HP loci. In four cases, pairs of *Eco*RI and *Hin*dIII fragments showed identical segregation patterns, and were presumably derived from the same endogenous locus. The segregation patterns of the loci were integrated into the chicken genomic map using the Mapmanager software (Manley, 1993) and showed that EAV-HP loci were present on several of the larger chromosomes, 1, 3, 4 and W. With the possible exception of one locus on the W chromosome, the positions of EAV-HP loci differed from those of the 12 EAV-0 loci mapped in the same population, indicating that they are quite distinct.

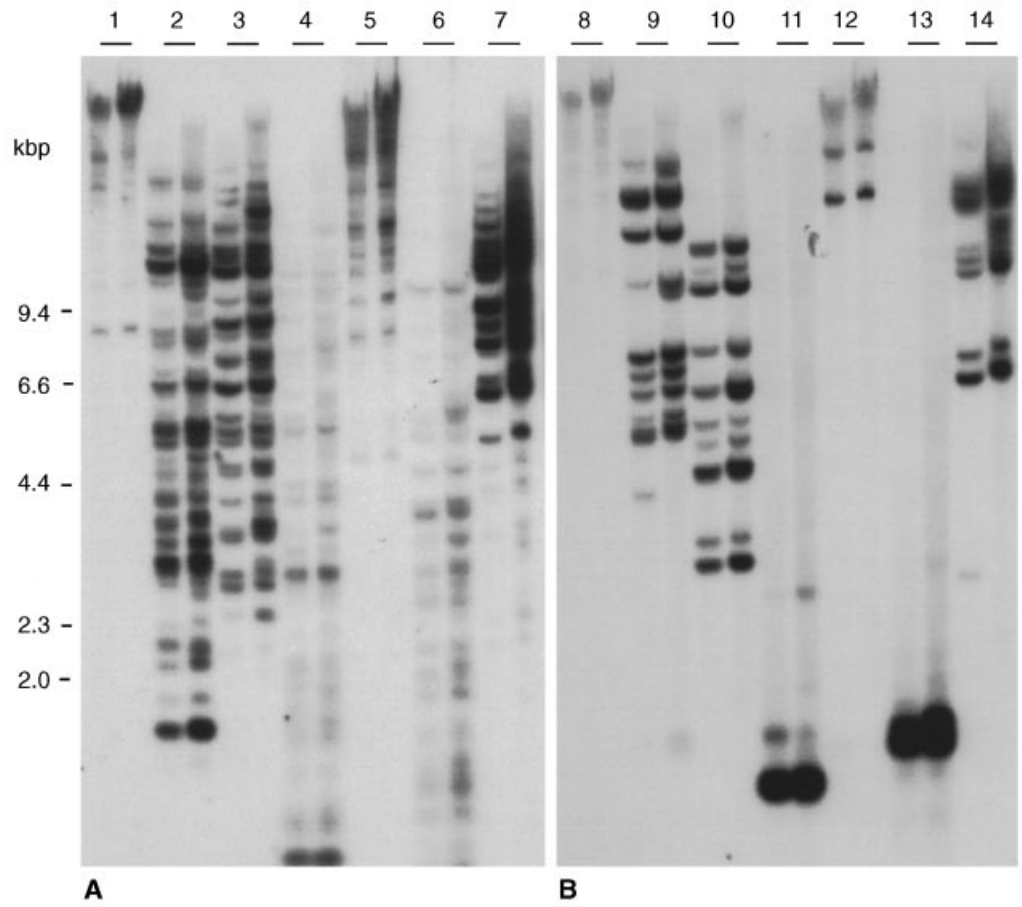

Fig. 2. Comparison of the hybridization pattern of EAV-0 and EAV-HP in chicken lines. Southern blots of genomic DNA digested with different restriction enzymes were probed with radiolabelled EAV-0 (A) and EAV-HP (B) probes. Pairs of lanes contain DNA from line N and line 15. Restriction enzymes used were: lanes 1 and 8, *Cla*I; 2 and 9, *Eco*RI; 3 and 10, *Hin*dIII; 4 and 11, *Msp*I; 5 and 12, *Xho*I; 6 and 13, *Taq*I; 7 and 14, *Bam*HI.

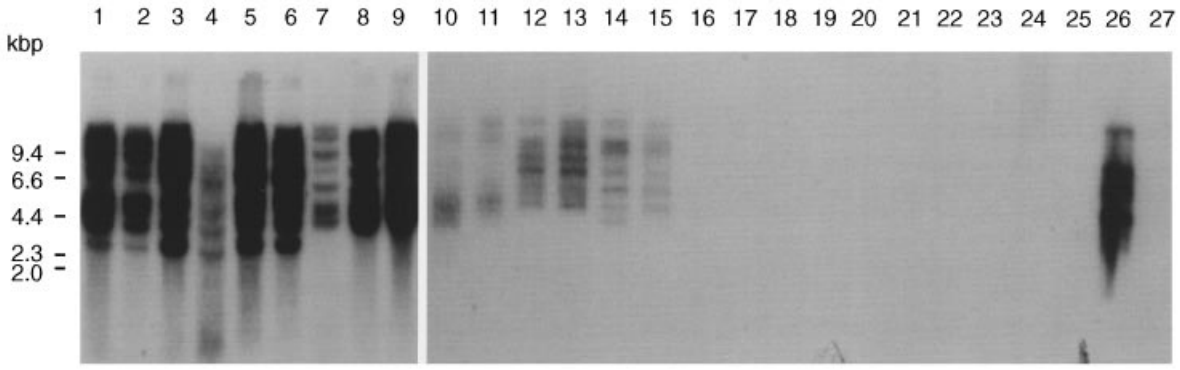

Fig. 3. Distribution of EAV-HP *env*-related sequences in the genomes of different species. Southern blots of *Eco*RI-digested genomic DNA separated by electrophoresis on 1% agarose gels were probed at a high stringency with a radiolabelled 548 bp EAV-HP *env* DNA fragment. DNA was derived from the following sources. Lanes 1–15, various chicken lines: 1 and 2, 15I; 3 and 4, C; 5 and 6, N; 7, 0; 8 and 9, 6_1; 10 and 11, Light Sussex; 12 and 13, 7_2; 14 and 15, Sykes Rhode Island Red; lane 16, ring-necked pheasant; 17, guinea fowl; 18, turkey; 19, Muscovy duck; 20, domestic duck; 21, mouse cell line L929; 22, human osteosarcoma cell line; 23, bovine MDBK cell line; (24, blank); 25, Japanese quail; 26, grey jungle fowl; 27, Vero cells.

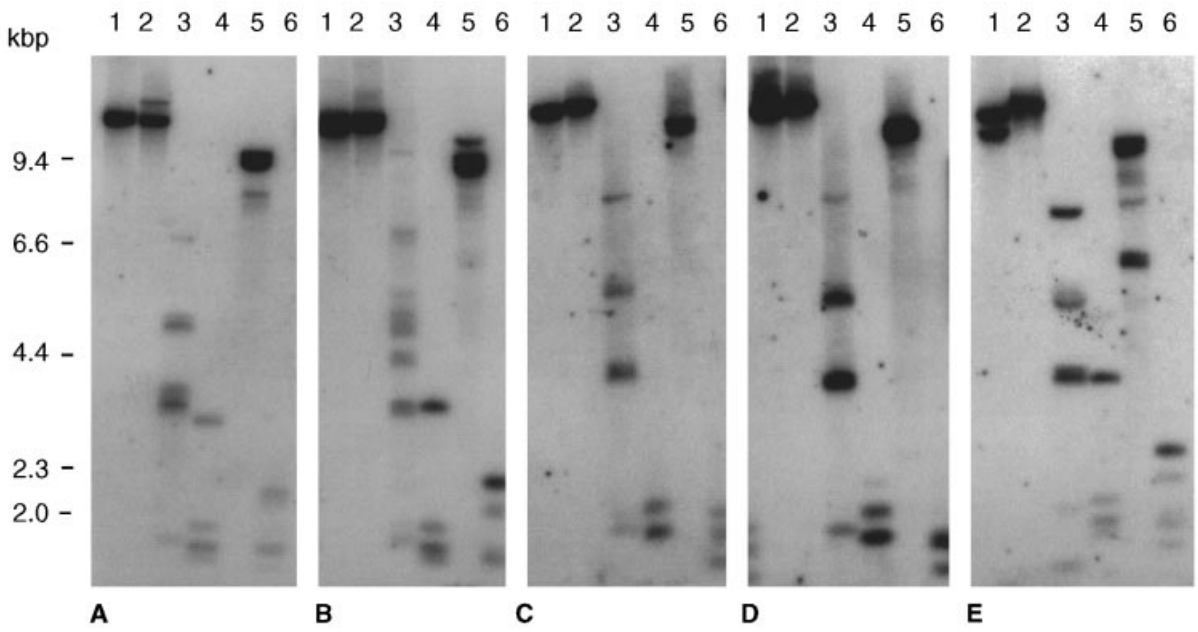

Fig. 4. Distribution of EAV-HP *env*-related sequences in five individual YAC clones, nos 58 (A), 62 (B), 63 (C), 75 (D) and 76 (E). Southern blots of YAC DNA digested with different restriction enzymes were probed with radiolabelled EAV-HP *env* DNA fragment. Lanes 1–6 contain DNA digested with *Eco*RI, *Bam*HI, *Rsa*I, *Alu*I, *Hin*dIII and *Msp*I, respectively.

Summaries of the current chicken genome map can be accessed through web sites at http://www.ri.bbsrc.ac.uk or http://www.poultry.mph.msu.edu.

Nucleotide sequence analysis

To determine the EAV-HP *env* sequence, the H3–37.1 PCR product from the genome of line 21 birds was cloned into pGEM-T and sequenced in both directions with oligonucleotide primers derived from vector and insert sequences. The nucleotide sequence was 97·4% identical to the HPRS-103 *env* gene and 69·1% identical to the E51 sequence. Inspection of the predicted open reading frame of the clone showed that the sequences of both the SU and transmembrane (TM) domains were nearly 96% identical to those of HPRS-103. The amplified region also included the 219 bp 'insertion' sequence, unique to HPRS-103 (Bai *et al.*, 1995), in the TM domain (Fig. 5).

Discussion

The avian genome harbours many families of ERV elements such as *ALVE*, CR-1, ART-CH and EAV-0 (Crittenden, 1991). The EAV-0 family has been shown to be very complex, being present in multiple copies and comprising the genetically distinct but closely related subfamilies EAV-0, E51, E13 and E33, which have approximately 70% sequence identity to each other. Although many of the EAV-0 loci present in the domestic chicken genome contain deletions within the *env* gene, E51 has been shown to have a full-length *env* sequence (Boyce-Jacino *et al.*, 1992). The presence of a large number of these endogenous loci, some of which may contain intact genes, provides the opportunity for recombination events to generate novel EAVs. Here we describe such a new subfamily of EAV, designated EAV-HP, which seems to have contributed in this manner towards the origin of a new exogenous avian retrovirus, HPRS-103, the prototype of the new subgroup J of ALV.

EAV-HP sequences in the chicken genome were detected initially by PCR with two HPRS-103 *env*-specific primers (H3 and H4). Products identical in size were obtained from genomic DNA prepared from both infected and uninfected chicken embryonic fibroblasts. Subsequent analysis with different sets of primers showed that a large region of the *env* gene of HPRS-103 was related to EAV-HP. However, the primers H5 and AJ1 did not amplify any sequences from the DNA of uninfected cells. As these two primers are derived from HPRS-103 sequences, PCR products obtained from infected cells with these primers are likely to be derived from exogenous ALV. We are currently trying to subclone full-length proviral DNA of EAV-HP from YAC clones; sequence analysis of these clones will provide the complete genomic organization of these novel ERVs.

In order to follow the distribution of this new subfamily of ERVs, we surveyed many species, including eight lines of chicken, the related species *G. sonneratii* and a number of other genera of birds and mammals, for the presence of specific EAV-HP *env*-related sequences. The genomes of all eight lines of

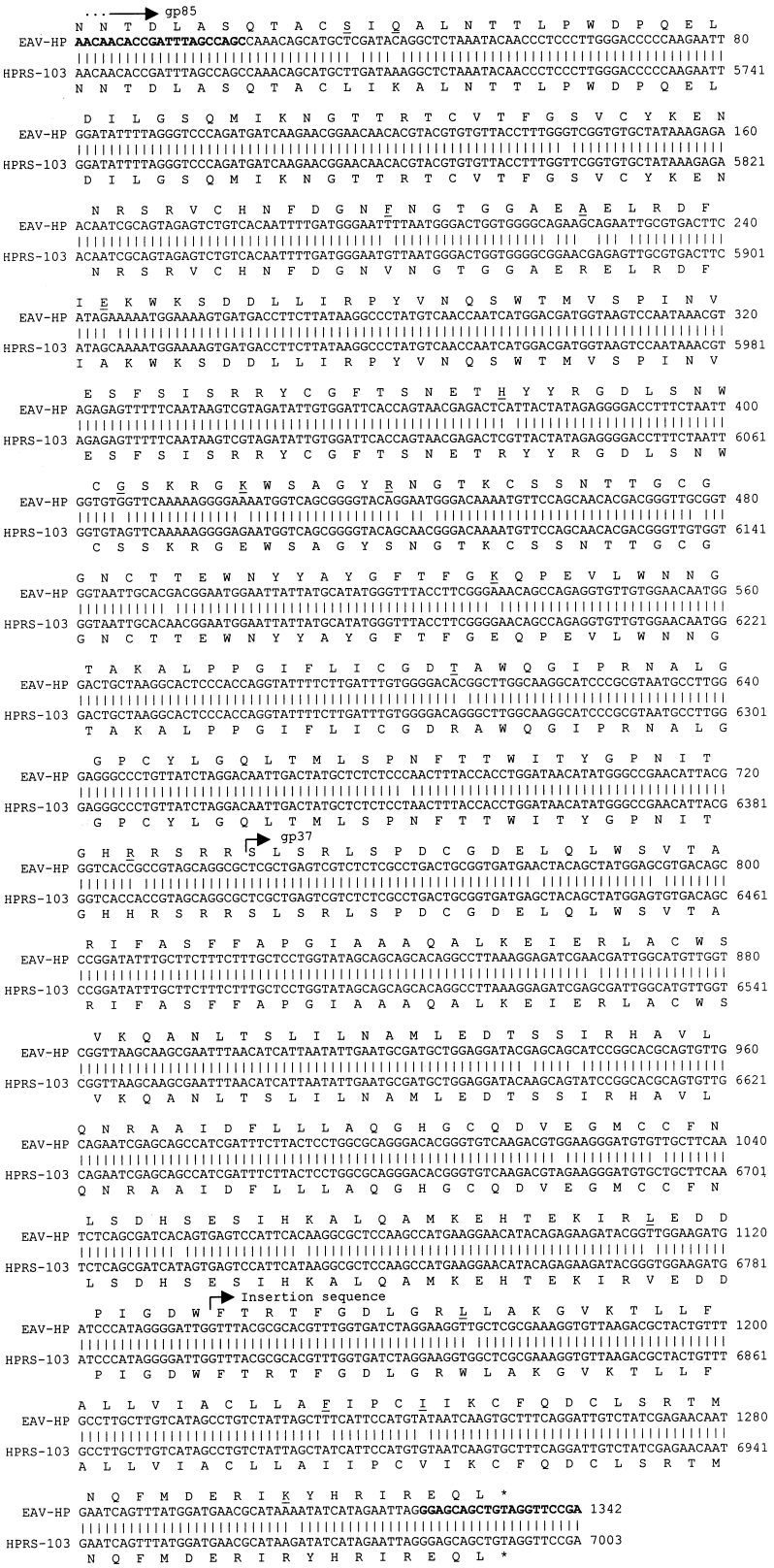

Fig. 5. Alignment of the *env* sequence of EAV-HP and HPRS-103. Alignment was done with the GAP program (GCG version 8.1, Genetics Computer Group). Vertical lines represent identity; amino acid changes are underlined. Residues 5661–7003 of the HPRS-103 sequence (accession no. Z46390) were used for the alignment. Primer sequences are shown in bold.

chicken and of *G. sonneratii* gave complex hybridization patterns with the probe, establishing the existence of EAV-HP elements in these birds. The patterns of restriction fragments were similar, suggesting conservation of these elements. DNA of birds such as ring-necked pheasant, Guinea fowl, turkey, Muscovy duck, domestic duck and Japanese quail, as well as the mammalian species examined, did not show any signals under the high hybridization stringency conditions used. Line 0 chicken and *G. sonneratii*, both lacking detectable RAV-0 elements, contained sequences of this new subfamily (Fig. 3). In this respect, EAV-HP elements showed a distribution very similar to that of EAV-0 (Resnick *et al.*, 1990) and may also represent an ancient retroviral family predating RAV-0. As our study did not include DNA from other *Gallus* species such as *G. varius* (green jungle fowl) and *G. lafayettei* (Ceylon jungle fowl), we cannot confirm whether the phylogenetic distributions of EAV-0 and EAV-HP are identical. Previous studies of the distribution of EAV-0 using locus-specific probes revealed that, although all *Gallus* species and domestic chickens harboured EAV-0, they all carried distinguishable complements of the proviral loci (Resnick *et al.*, 1990). The availability of full-length proviral clones of EAV-HP will provide opportunities to look at the nature of proviruses in different species of jungle fowl and domestic chicken.

The high sequence identity of the 1·3 kbp fragment of the EAV-HP *env* region to that of HPRS-103 strongly suggests that the latter was generated by recombination with these elements. HPRS-103 was isolated from meat-type birds, where it produces myeloid leukosis (Payne *et al.*, 1991), and is the prototype of the J subgroup of ALV. Although HPRS-103 virus has well-conserved *gag* and *pol* genes compared with other subgroups of ALV (96–97% identity), the *env* gene is highly diverged (only 40% overall identity), supporting the suggestion that this region was derived by recombination with EAV-HP elements rather than from changes in the *env* gene (Bai *et al.*, 1995). There is evidence to suggest that mammalian retroviruses such as murine leukaemia virus (Stoye & Coffin, 1987) and feline leukaemia virus (FeLV) (Chakrabarti *et al.*, 1994) have evolved by recombination with endogenous retroviral elements. However, until now there has been no evidence for such a mechanism for the evolution of oncogenic ALVs; HPRS-103 is the first avian example of such a virus evolved from an endogenous counterpart. Compared with other ALVs, the *env* gene of HPRS-103 contains a unique 'insertion' in the TM domain. The 3′ boundary of the recombination event that led to the generation of HPRS-103 is believed to be just downstream of this 'insertion' (Bai *et al.*, 1995). The sequence of the EAV-HP also shows this 'insertion', providing further evidence that HPRS-103 was derived by recombination with EAV-HP elements. Further sequence analysis of the EAV-HP elements downstream to this region should provide more information on the precise location of recombination. The HPRS-103 nucleotide sequence also reveals other distinct features, such as the presence of an E element in the 3′ non-coding region as well as a highly diverged LTR, possibly the result of multiple recombination events (Bai *et al.*, 1995). Molecular analysis of proviral clones of EAV-HP may reveal how and to what extent these elements have contributed to the origin of HPRS-103 and its unique features.

The envelope glycoproteins of ALV contain several neutralizing epitopes and are important in inducing protective immune responses. The main biological implication of the existence of endogenous *env*-related sequences in the genome is the potential induction of tolerance to HPRS-103 in birds where EAV-HP *env* sequences are expressed during the embryonic stages. We have preliminary evidence (not shown) to suggest that certain lines of chicken do express these sequences in embryonic tissues and such birds have been shown to develop persistent viraemia, suggestive of immunotolerence. Recent studies show evidence that expression of the *pol* gene of EAV-0 (Weissmahr *et al.*, 1997) is associated with the appearance of reverse transcriptase activity in cultures derived from chicken cells (Robertson *et al.*, 1997). At present we have no data to suggest that expression of EAV-HP *pol* gene results in a similar activity. Further characterization of the EAV-HP proviral clone will provide more information on this.

Finally, similar to mammalian retroviruses such as FeLV, high levels of antigenic variation, with extensive sequence changes in the hypervariable regions of the SU domain, have been reported among ALV subgroup J isolates (Venugopal *et al.*, 1998). In FeLV, it has been demonstrated that recombination between exogenous and endogenous sequences can contribute to antigenic diversity (Roy-Burman, 1995). It is not known whether the multiple copies of EAV-HP elements in the chicken genome are very diverse and could provide a potential source of genetic diversity to ALV subgroup J. However, endogenous virus *env* sequences, in comparison to those of exogenous ALVs, appear to be less divergent (Crittenden, 1991). Unlike the extensive sequence variation observed in the *env* genes of ALV subgroup J variants (Venugopal *et al.*, 1998), the sequences of the EAV-HP proviral DNA among different lines of chicken and the ancestral jungle fowl are very conserved. From this, it would appear that the antigenic diversity among ALV subgroup J isolates is most likely to result from mutations in the viral RNA and selection from immune pressure (Venugopal *et al.*, 1998), rather than from recombination events with multiple EAV-HP loci in the chicken genome.

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