Sequence of host-range determinants in the env gene of a full-length, infectious proviral clone of exogenous avian leukosis virus HPRS-103 confirms that it represents a new subgroup (designated J)

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A genomic DNA library was constructed, in a bacteriophage λ vector, from line 0 chick embryo fibroblasts (CEFs) infected with HPRS-103, an exogenous avian leukosis virus (ALV; envelope subgroup J) recently isolated from meat-type chickens. The library was screened at high stringency using a full length RAV-1 (subgroup A) proviral probe. From 10⁶ plaques, two clones which hybridized strongly to the RAV-1 probe were isolated; one contained a full-length copy of the proviral genome of HPRS-103 and the other contained a copy lacking the 5'-long terminal repeat (LTR) and part of gag. The relative strength of hybridization of RAV-1 and HPRS-103 clones, to RAV-1 probes representing different parts of the proviral genome, indicated that the gag and pol genes of HPRS-103 share a high level of identity with those of RAV-1 but that the env gene and the LTRs are considerably less well conserved. Infectious virus was recovered from CEFs transfected with the full-length clone, as detected by ELISA. The recovered virus appeared to be identical to HPRS-103 by electron microscopy and by Southern blotting of proviral DNA. The recovered virus was shown to be of the same subgroup as HPRS-103 by serum neutralization and receptor interference assays. Sequence analysis of the env gene of HPRS-103 shows that it differs considerably from the env genes of other ALV subgroups, particularly in the host range determinants, consistent with the finding that HPRS-103 represents a new subgroup (designated J).

The HPRS-103 strain of avian leukosis virus (ALV), isolated from meat-type chickens, is believed to represent a new envelope subgroup (designated J; Payne et al., 1991a, 1992b). The virus behaves as an exogenous virus, causing mainly myelocytic myeloid leukemia and nephromas, particularly in meat-type chicken strains (Payne et al., 1991b, 1992a). Unlike ALVs of other envelope subgroups, HPRS-103 was able to infect all chicken lines studied, as well as red jungle fowl and Sonnerat’s jungle fowl (Payne et al., 1992b). To facilitate studies on the tropism and oncogenicity of HPRS-103, we isolated a full-length proviral clone of HPRS-103.

HPRS-103 was propagated in primary chicken embryo fibroblasts (CEFs) freshly prepared from embryos (10- to 12-day-old) of the line 0 Leghorn chicken strain held as a specific pathogen-free (SPF) flock at the Institute for Animal Health. Line 0 of C/E phenotype is devoid of endogenous virus (ev) loci (Astrin et al., 1979). CEFs infected with HPRS-103 virus stock (Payne et al., 1991a) were passaged twice, at an interval of 3 days, then high-molecular-weight (HMW) genomic DNA was extracted (as described by Sambrook et al., 1989).

The presence of proviral DNA in genomic DNA from infected CEFs was monitored by Southern blotting using a [³²P]dCTP labelled RAV-1 DNA probe isolated from pRCAS (Hughes et al., 1987). HPRS-103 shows an identical profile to HPRS-F42 (subgroup A) ALV when digested with BamHI, as shown previously (Payne et al., 1991a), but profiles are very different from the subgroup A virus upon digestion with EcoRI, PstI, PvuII, SmaI, XbaI and XhoI (data not shown).

In an attempt to clone a full-length copy of the proviral genome, partially digested, size-fractionated HMW genomic DNA from cells infected with HPRS-103...
Fig. 1. Genetic organization of HPRS-103. (a) Comparison of genetic organization of genomes of HPRS-103 and RAV-1. DNA samples of recL5 (lane 1), recL7 (lane 2) and pRCAS (lane 3) were digested with BamHI, subjected to agarose gel electrophoresis and stained with ethidium bromide (slice e) or Southern blotted. The filter was cut into four slices (a to d) which were then hybridized with

(b)
was cloned into the bacteriophage \( \lambda \) vector, LambdaGEM-11 (Promega). From a library of \( 10^6 \) plaques, seven putative positive plaques were picked, purified and analysed by Southern blotting, using the RAV-1 DNA probe. Two of the clones (recL5 and recL7) hybridized strongly to the RAV-1 probe and those seen for HPRS-103-infected cell DNA (data not shown). Two of the clones showed weak hybridization with RAV-1 and \( \text{BamHI} \) profiles quite different from HPRS-103-infected cell DNA. The remaining three clones showed only extremely weak hybridization to RAV-1.

DNA from the strongly hybridizing clones, as well as the pRCAS control, was digested with \( \text{BamHI} \) and probed with partial RAV-1 sequences. These were derived from pRCAS cleaved with \( \text{BamHI} \) and represented: (i) upstream cellular flanking sequences, the 5' LTR and 5' \( \text{gag} \) sequences; (ii) \( \text{gag} \) sequences alone and (iii) \( \text{gag}--\text{pol} \) sequences. In addition, the RAV-1 gp85 sequences were amplified from pRCAS using specific oligonucleotides for use as an \( \text{env} \) probe. Like pRCAS, both of the HPRS-103 clones showed a 1-8 kbp \( \text{gag}--\text{pol} \) fragment (Fig. 1 a, lanes c1 to c3). Like pRCAS, recL5 has a 1-4 kbp \( \text{gag} \) fragment but the hybridizing fragment in recL7 is over 20 kbp (Fig. 1 a, lanes b1 to b3). The \( \text{env} \) gene of recL5 is located on a 6-4 kbp \( \text{BamHI} \) fragment but that of recL7 is located on a 3-9 kbp fragment (Fig. 1 a, lanes d1 to d3), a little larger than that of pRCAS (3-8 kbp). The LTR probe, which hybridizes to two LTR fragments in pRCAS (3-8 kbp, the same as \( \text{env} \), and 4-6 kbp), hybridizes to two fragments in recL5 (6-4 kbp, the same as \( \text{env} \), and 3 kbp) but to only one fragment in recL7 (3-9 kbp, the same as \( \text{env} \); see Fig. 1 a, lanes a1 to a3). To facilitate more detailed mapping, the HPRS-103 sequences from recL5 and recL7 were subcloned from the bacteriophage \( \lambda \) vectors into the Bluescript plasmid vector pBK II KS(−). From the results of restriction digests of the phage and plasmid clones (data not shown), maps for recL5 and recL7 were constructed (Fig. 1 b). RecL5 appears to be a full-length copy of the proviral genome of HPRS-103, with 5' cellular flanking sequence and the 5' LTR on a 3 kbp \( \text{BamHI} \) fragment. The \( \text{gag} \) and 5' \( \text{pol} \) sequences are carried on \( \text{BamHI} \) fragments of 1-4 and 1-8 kbp, while a 6-4 kbp \( \text{BamHI} \) fragment contains the 3' \( \text{pol} \) sequences, \( \text{env} \), and the 3' LTR and 3' cellular flanking sequence. RecL7 lacks the 5' LTR and part of \( \text{gag} \) but the \( \text{pol} \) and \( \text{env} \) genes as well as the 3' LTR appear to be intact.

As the HPRS-103 clone in recL5 appeared to be full length, the cloned DNA was transfected into CEFs using lipofectin (BRL; Felgner et al., 1987), to see if it was capable of directing the production of infectious virus. Expression of \( \text{Gag} \) proteins in lysates of transfected cells (line 0 CEFs) was monitored by ELISA. Transfected but not control CEFs showed significant \( \text{Gag} \) expression (Table 1). To see if infectious virus was recovered from the transfected CEFs, fresh CEFs were incubated with the supernatants from the transfected (or mock-transfected) cells. Those CEFs incubated with the supernatants from the transfected, but not from the mock-transfected CEFs also showed significant \( \text{Gag} \) expression.

### Table 1. Recovery of infectious virus from HPRS-103 genomic DNA clone recL5*

<table>
<thead>
<tr>
<th>Amount of recL5 DNA used (µg)</th>
<th>ELISA absorbance value</th>
<th>Infection with recovered virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1:14</td>
<td>0:97</td>
</tr>
<tr>
<td>10</td>
<td>1:10</td>
<td>1:09</td>
</tr>
<tr>
<td>0</td>
<td>0:06</td>
<td>0:01</td>
</tr>
</tbody>
</table>

* CEFs freshly prepared from 10-day-old line 0 chicken embryos were cultivated up to 80% confluence in 4.5 cm diameter dishes and washed in Optimum 1 medium (Gibco) just before transfection. Afterwards 2 ml of Optimum 1 was added to three dishes and incubated at 38 °C. SfiI-digested recL5 recombinant bacteriophage \( \lambda \) DNA (5 or 10 µg) was diluted into 50 µl of distilled water and gently mixed with diluted lipofectin (BRL; 30 µg in 50 µl of water) and held at room temperature for 15 min. The DNA-lipofectin complex was transferred to the tissue culture wells. The dishes were cultured at 38 °C for 3 days and then subcultures were made. After 3 days, supernatants from the above transfected and mock-transfected subcultures were used to infect fresh CEFs. Supernatants from the freshly infected CEF subcultures were harvested and stored at −20 °C. Lysates, prepared from transfected and infected CEFs by addition to washed cells of NP40 (0.1% in PBS), were used to coat 96-well plates. ALV Gag proteins were detected in an ELISA test (Payne et al., 1991 a) by chicken anti-ALV p27 polyclonal antibodies followed by a commercial, peroxidase-conjugated, anti-chicken IgG. Results are presented as ELISA absorbances. Cell lysates from mock-transfected and infected cells were used as negative controls.

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C. J. Payne et al. (1987) recovered the proviral DNA clone recL5 from the HPRS-103 clone, and cloned it into the bacteriophage \( \lambda \) vector LambdaGEM-11 (Promega). From a library of \( 10^6 \) plaques, seven putative positive plaques were picked, purified and analysed by Southern blotting, using the RAV-1 DNA probe. Two of the clones (recL5 and recL7) hybridized strongly to the RAV-1 probe and showed \( \text{BamHI} \) restriction enzyme profiles similar to those seen for HPRS-103-infected cell DNA (data not shown). Two of the clones showed weak hybridization with RAV-1 and \( \text{BamHI} \) profiles quite different from HPRS-103-infected cell DNA. The remaining three clones showed only extremely weak hybridization to RAV-1.

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Table 2. Assay of neutralization of rHPRS-103 by sera specific for ALVs of subgroups A to D and J*

<table>
<thead>
<tr>
<th>Antiserum against:</th>
<th>Neutralization of rHPRS-103*</th>
<th>ELISA (absorbance value)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus</td>
<td>Subgroup</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAV-1</td>
<td>A</td>
<td>1.19</td>
<td>-</td>
</tr>
<tr>
<td>RAV-2</td>
<td>B</td>
<td>1.21</td>
<td>-</td>
</tr>
<tr>
<td>RAV-49</td>
<td>C</td>
<td>1.18</td>
<td>-</td>
</tr>
<tr>
<td>RAV-50</td>
<td>D</td>
<td>1.17</td>
<td>-</td>
</tr>
<tr>
<td>HPRS-103</td>
<td>J</td>
<td>0.04</td>
<td>+</td>
</tr>
<tr>
<td>Negative serum</td>
<td>-</td>
<td>1.19</td>
<td>-</td>
</tr>
</tbody>
</table>

* The microneutralization assay has been described previously (Fadly et al., 1989). Virus growth, detected by Gag-specific ELISA, is indicative of resistance to neutralization. Combinations of antisera only neutralized when HPRS-103 antiserum was present (data not shown).

Table 3. Receptor interference between rHPRS-103 and RSV pseudotypes of ALSV subgroups A to D and J in line 0 C/E CEFs*

<table>
<thead>
<tr>
<th>Challenge virus and subgroup</th>
<th>Control cells</th>
<th>rHPRS-103-infected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV (RAV-1) A</td>
<td>83</td>
<td>70</td>
</tr>
<tr>
<td>RSV (RAV-2) B</td>
<td>76</td>
<td>90</td>
</tr>
<tr>
<td>RSV (RAV-49) C</td>
<td>79</td>
<td>79</td>
</tr>
<tr>
<td>RSV (RAV-50) D</td>
<td>49</td>
<td>63</td>
</tr>
<tr>
<td>RSV (HPRS-103) J</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Results are mean of counts of foci in duplicate dishes.

The presence of HPRS-103 proviral DNA in the CEFs incubated with the recovered virus was shown by Southern blotting (data not shown). Electron microscopy of those cells infected with the recovered virus showed typical C-type retroviral particles (data not shown). The subgroup of the recovered virus (rHPRS-103) was confirmed by serum microneutralization tests (Table 2; Fadly et al., 1989) and RSV pseudotype interference assays (Table 3; Payne et al., 1991a), both of which showed it to be of the same subgroup (J) as HPRS-103.

The relationship of RAV-1 sequences to those of HPRS-103 can be inferred from the relative strength of the hybridization signals seen in Fig. 1. The 1.8 kbp BamHI fragments of rRCAS, recL5 and recL7 are present at similar levels as shown by ethidium bromide staining (Fig. 1a, lanes e1 to e3) and all three fragments present a hybridization signal of similar intensity when probed with the 1.8 kbp gag-pol RAV-1 probe (Fig. 1a, lanes c1 to c3). The same is true for molar equivalents of the gag gene-containing 1.4 kbp BamHI fragments of rRCAS and recL5 and the 20 kbp fragment of recL7 when probed with the 1.4 kbp RAV-1 gag probe (Fig. 1a, lanes b1 to b3). The hybridization signal obtained with the rRCAS gp85 probe at high stringency is, however, much lower for the 6.4 kbp and 3.9 kbp BamHI fragments of recL5 and recL7, respectively, than for the 3.8 kbp rRCAS fragment (Fig. 1a, lanes d1 to d3). Similarly, the hybridization signals obtained with the LTR-containing fragments of recL5 (6-4 and 3 kbp) and recL7 (3-9 kbp) are much lower than those obtained with the LTR fragments of rRCAS (3-8 and 4-6 kbp; Fig. 1a, lanes a1 to a3). On the basis of these results for hybridization at high stringency, it would therefore appear that the gag and pol genes of HPRS-103 share a high level of sequence identity with the RAV-1 genes but that the env gene and the LTRs are considerably diverged from their RAV-1 equivalents.

The central region of the gp85 subunit of the env gene of avian leukemia-sarcoma viruses (ALSV) has been shown to comprise the host range determinants involved in receptor specificity (see Dorner et al., 1985; Bova et al., 1986). As the env gene of HPRS-103 seemed so diverged from that of RAV-1, sequence analysis of the gp85 coding region was performed so that the host range determinants of the subgroup J virus could be compared with those of other subgroups. Multiple sequence alignment revealed that the sequence encoding the gp85 domain of HPRS-103 is 93 to 126 bases shorter than those of viruses from other subgroups (with five deletions of nine or more bases and a 27 base deletion) and it only has 40% overall average identity to the corresponding sequences of subgroups A to E. (In comparison, the gp85-encoding sequences of viruses from subgroups A to E show 80 to 85% identity to each other.)

Comparisons of the derived amino acid sequence of gp85 with those of other ALSV subgroups are shown in Fig. 2. It is clear from a dendrogram showing the overall relationship of gp85 amino acid sequences that the HPRS-103 gp85 is well diverged from those of the other ALSV subgroups (Fig. 2a). The N-terminal 40 residues are relatively well conserved, as is a stretch of 40 residues near the C terminus (Fig. 2b). The central region, which spans the entire region identified as specifying receptor tropism (Dorner et al., 1985; Bova et al., 1986) is well diverged, not only in regions where high (hr1, hr2) or low (vr1, vr2 and vr3) variability exists between other subgroups but also in regions separating the variable regions, where the other subgroups show relatively high conservation (Dorner et al., 1985). The regions of HPRS-103 gp85 equivalent to those identified as contributing the major ALSV host range determinants (hr1 and hr2; Dorner et al., 1985; Bova et al., 1986) are highly diverged and contain a nine residue insertion and an eight residue deletion (at the C terminus), respectively (Fig. 2b). Even though hr1 and hr2 are highly variable between subgroups, there are a number of positions conserved.
Fig. 2. Comparison of gp85 amino acid sequences from ALSVs of subgroups A (ALVCG; Bieth & Darlix, 1992), B (REALRAVE; Bova et al., 1986), C (RERSV6; Schwartz et al., 1983), D (ALRSRD; Kihira, 1992), E (REALRG02; Dorner et al., 1985) and J (HPRS-103). All sequences were obtained from the EMBL database under the above names, except for that of HPRS-103, which is reported here, and were aligned using PILEUP. (a) The relationship of the gp85 sequences as determined by PILEUP is displayed using the FIGURE program and (b) the alignments are displayed using PRETTYBOX (Sequence Analysis Software Package 7.2, Genetics Computer Group, Madison, Wisconsin, USA; Devereux et al., 1984). Residues boxed in black are those which are identical to the majority of the aligned sequences. Shaded residues indicate conservative changes from the majority sequence. Dots indicate deletions. Regions previously identified as variable between subgroups A to E and potentially involved in host range determination are indicated by horizontal bars.
between subgroups A to E (of the 43 to 51 amino acids that make up hr1, eleven are conserved between all subgroups and six out of the 27 to 30 amino acids are conserved in hr2). Of those residues in hr1 and hr2 that are conserved between subgroups A to E, only one (of eleven) in hr1 and two in hr2 (of six) are also conserved in HPRS-103. At four more positions, conserved in hr1 and hr2 between subgroups A to E, the changes found in HPRS-103 are conservative and at seven other positions in hr1 and hr2, HPRS-103 has the same sequence as the majority of the other subgroups. Vrl appears to be in hrl and hr2, HPRS-103 has the same sequence as the HPRS-103 are conservative and at seven other positions in hrl and hr2 between subgroups A to E, the changes found in HPRS-103. At four more positions, conserved in hrl and hr2 (of six) are also conserved in HPRS-103 (and there is a conservative change at another such position).

It appears that while there may be major changes to the primary and secondary protein structure of the host determinants of HPRS-103 gp85 compared to those of other subgroups, a number of common structural features may remain, which is supported by secondary structure predictions. In a recent analysis of the secondary structure of gp85 of subgroups A, B, C and E, it was shown that the central part of hr2 of RAV-1 (subgroup A) is the most hydrophilic region of the molecule but that hr1 is much less hydrophilic, particularly at the N terminus (Valsesia-Wittmann et al., 1994). We compared the hydrophilicity of HPRS-103 gp85 with that of another subgroup A virus (EMBL: ALVCG; ALV-RSA, Bieth & Darlix, 1992) and found that hr2 has a similar hydrophathy profile in both viruses, being quite hydrophilic, particularly at the N terminus (data not shown). The hr1 differs between the two viruses, however, in that the N terminus in HPRS-103 is more hydrophilic than the C terminus with the converse being true in ALVCG (data not shown). The structure predicted for hr1 in both viruses, however, is similar in that it consists of α-helix followed by β-sheet followed in turn by α-helix (data not shown), as also indicated for RAV-1 (Valsesia-Wittmann et al., 1994).

In studies using recombinants between viruses of subgroups B and E, Dorner & Coffin (1986) showed that hr1, hr2, and to a lesser extent vr3, play the major role in specifying receptor tropism. A recombinant with hr1 and vr3 of subgroup B but hr2 of subgroup E had dual specificity but the conversion of either hr1 or vr3 to subgroup E type resulted in a virus specific for subgroup E receptor only. In a study to locate the determinants of subgroup D viruses responsible for mammalian cell tropism, it was shown that only hr1 sequences from subgroup D could confer on subgroup B an ability to infect mammalian cells (Bova-Hill et al., 1991). The sequence variation between HPRS-103 and viruses of other subgroups suggests that determinants for subgroup J specificity could be present at several different sites within the central portion of gp85. Analysis of the relative importance of those sites will require the analysis of the phenotype of recombinants, a task that will be facilitated by the existence of the infectious clone. The broad host range demonstrated for HPRS-103 amongst chicken lines (Payne et al., 1992b) means that derivatives of the proviral clone may also prove to be useful tools for the delivery of sequences to chickens or to cells derived from them. The infectious nature of the clone also means that it will be possible to investigate the role of HPRS-103 sequences in tissue tropism and oncogenesis.

We thank Professor Robin Weiss for his support and helpful discussions.

References


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