A new family of avian retroviral endogenous sequences designated ev/J or EAV-HP has been identified recently. Here an additional avian ev/J 4.1 endogenous sequence, ev/J 4.1 Rb, is reported. ev/J 4.1 Rb has the most extensive amino acid identity ever described for an endogenous envelope protein with the ALV-J avian leukosis virus. Here, we also demonstrate that ev/J 4.1 Rb functionally pseudotypes murine leukemia viros and leads to a complete reciprocal interference with ALV-J envelopes. This is the first demonstration of such a high level of envelope interference between endogenous and exogenous avian retroviruses. Our results provide additional clues on the co-evolution of retroviral sequences among vertebrates.

Two laboratories have recently reported the identification of the new ev/J (or EAV-HP) family of chicken endogenous retroviral sequences (Benson et al., 1998; Smith et al., 1999). Sequence comparisons in the envelope genes have revealed over 95 % identity between ev/J and the avian leukosis virus subgroup J (ALV-J). This level of sequence identity, suggesting a common origin for these two viral and cellular genes, is so far the highest one described for an endogenous retroviral sequence and the ALV-J env gene. Subgroup J ALVs were isolated in 1989 from meat-type birds in commercial flocks (Payne et al., 1991) and were found to induce myeloid rather than lymphoid leukemia. Sequencing of the HPRS-103 provirus, the ALV-J prototype, revealed extensive env gene differences with ALV-A to -E (Bai et al., 1995). Although the ev/J 4.1 endogenous sequence has been found to harbour a full Env ORF (Ruis et al., 1999), neither in vivo nor in vitro expression of ev/J 4.1 Env glycoproteins has ever been described, leaving unanswered questions about the ev/J 4.1 Env potential functionality. One of the key functions of exogenous retrovirus envelopes is the binding of cell ligands that act as receptors for virus entry. Since the ALV-J cellular ligand is unknown and since ALV-J gp85 Env precursor cannot be aligned with ALV-A to -E to predict specific receptor-binding domain key sequences, we tested the ev/J 4.1 envelope for functionality and evaluated potentially interfering receptor recognition between the ev/J 4.1 and ALV-J Env proteins. Using pseudotyped murine leukemia virus (MLV) virions, we have demonstrated that the ev/J 4.1 gene Env product is functionally expressed at the cell surface and mediates infection. Furthermore, we have shown that ev/J 4.1 Env belongs to the same interference group as the ALV-J subgroup Env protein.

We generated an ev/J 4.1 env expression plasmid. For this, total genomic DNA was isolated from the blood of a meat-type strain chicken and the env gene was amplified by PCR with ev/J-specific primers under conditions similar to those described by Ruis and colleagues using the Herculase DNA polymerase (Stratagene) (Ruis et al., 1999). The expected 2.2 kbp PCR product was purified (QIAquick gel extraction kit; Qiagen) and sequenced on both strands in the region corresponding to the env gene. This PCR product was then cloned into a pGEM-T easy vector (Promega). One clone, ev/J 4.1 Rb, was selected and sequenced. The ev/J 4.1 Rb sequence matched that of the PCR product and was found to be different from the published ev/J 4.1 prototype env gene cloned from DF-1 cells (ev/J 4.1 DF; GenBank accession no. AF125528) (Benson et al., 1998). The amino acid sequence deduced from ev/J 4.1 Rb probably corresponds to the natural parental isolate since it was closer in identity to the Env protein of two ALV-J reference strains, HPRS-103 (96·74 %) and Hc1 (95·65 %), than was ev/J 4.1 DF (94·25 % and 93·45 %, respectively) (Fig. 1). The 1828 bp SnaBI–MluI fragment containing the complete ev/J 4.1 Rb env sequence was excised, blunt-ended and cloned into pcDNA3 at the EcoRV site. The junctions of this pEEJ4.1 expression vector, also expressing the neomycin selection marker, were verified by DNA sequencing. The expression of ev/J 4.1 Rb env in TelCeB6 cells was verified by RT-PCR with the previously reported primer pair EAV-SD and 103-ER (Sacco & Venugopal, 2001) (data not shown).

In order to evaluate the ev/J 4.1 Env functionality, we generated recombinant MLV LacZ pseudotypes bearing ev/J 4.1 Rb or ALV-J Env proteins, after transfection of the
pEEJ4.1 or pZeoHc1 env expression vectors in TELCeB6 cells. pZeoHc1 is an expression vector of the exogenous ALV-J ADOL-Hc1 Env (kindly provided by Dr H. Hunt) (Hunt et al., 1999). This construct is similar to pEEJ4.1 as both are derived from a pcDNA vector. Virus harvests from TELCeB6 transfected cells were tested for transduction of the lacZ gene in DF-1 cells. DF-1 is a chicken fibroblast cell line (ATCC no. CRL-12203) developed from an ev-0 embryo susceptible to infection by ALVs, including ALV-J (Himly et al., 1998, Hunt et al., 1999). Individual wells in 24-well dishes were plated with 70,000 DF-1 cells and challenged with fivefold serial dilutions of fresh cell-free supernatants (0.45 μm pore-size filtered cell supernatants) from transfected TELCeB6 cells. Cell-free infections were ensured by using 0.45 μm pore-size filtered cell supernatants. At 48 h post-infection, DF-1 lacZ-transduced cells were stained with X-Gal solution as previously described (Denesvre et al., 1995). Infection was observed with the two avian retroviral Env pseudotypes, LacZ (ev/J4.1 Rb) and LacZ (Hc1), as well as with the positive control pseudotype LacZ (VSV-G) (vesicular stomatitis virus type G), obtained by using the pMDG construct (Ory et al., 1996). LacZ
(ev/J 4.1 Rb) and LacZ (Hc1) titres on DF-1 cells ranged around 4000 and 7000 infectious units ml\(^{-1}\), respectively (Table 1). These results show that ev/J 4.1 Env is correctly expressed at the cell surface. Therefore, the low identity in the signal peptide region between ev/J 4.1 and ALV-J Env products (48 %) does not affect the translocation into the endoplasmic reticulum. In addition, functional ev/J 4.1 Rb and Hc1 envelopes were efficiently incorporated into MLV-based virions and able to promote infection, as previously described with the Env of other ASLV subgroups (Adkins et al., 2000; Landau & Littman, 1992).

LacZ MLV pseudotypes were further used to investigate the interference patterns of the ev/J 4.1 Rb and HPRS-103 Env proteins. LacZ (ev/J4.1 Rb) and LacZ (Hc1) were plated on DF-1 cells pre-infected with either HPRS-103 or RAV-1, the prototype of the ALV-A subgroup. Table 1 summarizes the relative infectivity on pre-infected DF-1 cells compared with the uninfected parental cells. As expected, LacZ (VSV-G) infection was not blocked on any of the DF-1 cells. Pre-infection of DF-1 cells with HPRS-103, but not with RAV-1, completely blocked infection with LacZ (Hc1), confirming that Hc1 and HPRS-103 Env belong to the same J subgroup of interference. Similar results were obtained with LacZ (ev/J4.1 DF), indicating that Hc1 and ev/J 4.1 Rb Env belong to the same group of Env interference and thus use the same receptor. In order to monitor whether ‘cross-interference’ between ALV-J envelopes and ev/J 4.1 Rb was specific to a particular isolate or not, we tested the interference of LacZ (ev/J4.1 Rb) on DF-1 cells infected with a new French ALV-J isolate, INRA-7. Alignment of the predicted amino acid sequence of INRA-7 with other ALV-J Env proteins showed that INRA-7 Env harbours 92-98 % and 92-25 % amino acid sequence identity with HPRS-103 Env and Hc1.

![Protein sequence comparison of ALV-J and ev/J 4.1 Env proteins](Fig. 1)
Table 1. Infectious and interfering properties of ALV-J and ev/J 4.1 envelopes of MLV pseudotypes on parental and ALV-A or -J chronically infected DF-1 cells.

<table>
<thead>
<tr>
<th>Target cell/virus</th>
<th>LacZ (ALV-J Hc1)†</th>
<th>LacZ (ev/J 4.1)†</th>
<th>LacZ (VSV-G)†</th>
<th>LacZ (H20)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF-1</td>
<td>7050</td>
<td>4790</td>
<td>22 460</td>
<td>0</td>
</tr>
<tr>
<td>DF-1/HPRS-103§</td>
<td>0</td>
<td>0</td>
<td>48 400</td>
<td>0</td>
</tr>
<tr>
<td>DF-1/RAV-1§</td>
<td>7100</td>
<td>8240</td>
<td>35 000</td>
<td>0</td>
</tr>
</tbody>
</table>

*Representative titres of three to four independent experiments are shown as number of blue cells ml⁻¹.
†Recombinant LacZ pseudotypes were produced by transient transfection of env expression plasmids for ALV-J Hc1, ev/J 4.1 and VSV-G into TELCeB6 cells.
‡LacZ (H20) was produced by mock transfection into TELCeB6 cells.
§HPRS-103 and RAV-1 are ALV-J and ALV-A strains, respectively.

Env, respectively (Fig. 1). Chronic infection of DF-1 cells with INRA-7 ALV-J isolate also blocked entry of LacZ (ev/J 4.1 Rb) (data not shown).

The pEEJ4.1 plasmid was used to generate DF-1 cells constitutively expressing the ev/J 4.1 Rb. After transfection of DF-1 cells with pEEJ4.1, G418-resistant cells were selected using 800 µg geneticin (Gibco) ml⁻¹. G418-resistant clones were isolated and analysed for cell-surface expression of ev/J 4.1 Env by flow cytometry using a polyclonal chicken antiserum to HPRS-103. Two DF-1 clones, clones 2 and 4, were selected for their high level of Env cell-surface expression. In order to test the interference properties of the ev/J Env protein, the two DF-1 ev/J clones were challenged with LacZ virion pseudotypes as described above. The number of LacZ-positive cells was counted 48 h post-infection (Table 2). No blue cells were detected after challenging infection with LacZ (Hc1) or Lac Z (ev/J 4.1 Rb), thus demonstrating that overexpression of ev/J 4.1 Rb Env alone mediates complete auto- and cross-interference with ev/J 4.1 Rb Env and ALV-J Hc1 Env, respectively.

In this work, we used MLV pseudotypes bearing ALV-J-type envelope proteins. Production of RSV pseudotypes bearing ALV-J Env after infection with HPRS-103 of Env-deficient R(−)Q Rous sarcoma virus (RSV)-transformed non-producer quail cells (Crittenden et al., 1979) was inefficient, as these cells are not susceptible to HPRS-103 (Venugopal et al., 1998). The MLV pseudotyping system described here has the advantage of resulting from the transfection of a unique plasmid as opposed to other previously described systems (Adkins et al., 2000) and may thus be used to obtain a stable pseudotype-producing cell line (Cosset et al., 1995). Our model also generated supernatants that are readily titrated and that homogeneously contain pseudotyped virions, devoid of the parental infectious virus carrying the Env protein.

ev/J 4.1 is the only EAV-HP or ev/J provirus identified from chicken genomes that carries a complete env gene. However, no protein expression of this provirus has been described to date. We have shown here that ev/J 4.1 Rb ORF expression leads to production of a functional Env protein. Like other retroviral signal peptides, the signal peptides of ev/J 4.1 and ALV-J contain an N-terminal region rich in charged amino acids and a central hydrophobic core followed by small polar amino acids (Ellerbrok et al., 1992). Our observation of

Table 2. Infectious and interfering properties of ALV-J and ev/J 4.1 envelopes of MLV pseudotypes on DF-1 cells stably expressing the ev/J 4.1 envelope.

<table>
<thead>
<tr>
<th>Target cell</th>
<th>LacZ (ALV-J Hc1)†</th>
<th>LacZ (ev/J 4.1)†</th>
<th>LacZ (VSV-G)†</th>
<th>LacZ (H20)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF-1</td>
<td>7775</td>
<td>10 850</td>
<td>21 250</td>
<td>0</td>
</tr>
<tr>
<td>DF-1 ev/J 4.1 Env clone 2</td>
<td>0</td>
<td>0</td>
<td>43 000</td>
<td>0</td>
</tr>
<tr>
<td>DF-1 ev/J 4.1 Env clone 4</td>
<td>0</td>
<td>0</td>
<td>48 625</td>
<td>0</td>
</tr>
</tbody>
</table>

*Representative titres of two to four independent experiments are shown as number of blue cells ml⁻¹.
†Recombinant LacZ pseudotypes were produced by transient transfection of env expression plasmids for ALV-J Hc1, ev/J 4.1 and VSV-G into TELCeB6 cells.
‡LacZ (H20) was produced by mock transfection into TELCeB6 cells.
the rather low sequence identity between the ev/J 4.1 and ALV-J Env signal peptides confirms that conservation of these general features, rather than of strict amino acid sequences, can also predict the avian Env signal peptide functionality.

The fact that the endogenous ev/J 4.1 Rb and exogenous ALV-J Env exhibit complete and fully reciprocal interference to superinfection indicates that they share the same receptor(s) on DF-1 cells. On the basis of their interference properties and amino acid divergences with ALV-E endogenous envelope sequences, we propose to classify ev/J 4.1 endogenous virus Env into the ALV-J subgroup. This subgroup will therefore be the first ALV subgroup described that includes both endogenous sequences and exogenous viruses. The high sequence homologies and similar functional properties observed between ev/J 4.1 and ALV-J Env most likely indicate the recent emergence of exogenous infectious ALV-J viruses after the recombinatorial insertion of ev/J endogenous sequences.

The complete in vitro interference described here is reminiscent of the MLV interference observed with the murine ecotropic (Fv-4) and polytropic (rmuc) endogenous retroviral sequences (Ikeda & Sugimura, 1989; Lyu et al., 1999). However, unlike the MLV ecotropic env-like Fv-4 resistance gene, ev/J 4.1 encodes an Env protein capable of driving virion infection similar to endogenous MLV polytropic sequences (Lavignon et al., 1994). Whether the ev/J 4.1 loci confer in vivo resistance for ALV-J infection or ALV-J-induced disease is still not known and becomes of importance in view of the recent ALV-J epidemics. Previous attempts to detect ev/J 4.1 env mRNA either from an ev0 chicken embryo cDNA library by RT-PCR or by Northern blot did not succeed (Ruis et al., 1999). Nevertheless, microarrays containing 1000 non-redundant selected cDNAs, used to examine host-cell gene expression after Marek’s disease avian herpesvirus infection, revealed the expression of a mRNA encoding an ALV-J Env protein (Morgan et al., 2001). This mRNA may have derived from the expression of an ev/J 4.1 sequence. Therefore, determining the conditions of expression of ev/J 4.1 sequences and their effect on further virus infection will help to elucidate the impact of this sequence in the chicken genome.

ACKNOWLEDGEMENTS

We are indebted to K. Venugopal for the HPRS-103 strain and to H. Hunt for pZe0Hc1. We thank F.-L. Cosset and J.-C. Pagès for kindly providing us with TELCeb6 cells and plasmids, and Yves Le Vern and Dominique Kerboeuf for technical help in flow cytometry. We are very grateful to M. Sitbon and J.-C. Pagès for critical reading of the manuscript. This work was supported by a grant from Biotechnocentre, Conseil de la Région Centre (France).

REFERENCES


