Selection of a Subgroup A Avian Leukosis Virus [ALV(A)] Envelope Resistant to Soluble ALV(A) Surface Glycoprotein

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The host developing resistance to retroviral infection is believed to be a major force in the evolution of multiple receptor usage by retroviruses. The avian leukosis–sarcoma virus (ALV) group of retroviruses provides a powerful system for studying the envelope–receptor interactions involved in retrovirus entry; different members of this group of closely related viruses use distinct cellular receptors. Analysis of the ALV envelope subgroups suggests that the different ALVs evolved from a common ancestor by mutations in the env gene. Cells and animals that express subgroup A ALV envelope glycoproteins are highly resistant to ALV(A) infection due to receptor interference. In this study, we tested whether expression of a soluble form of subgroup A surface glycoprotein (SU) would result in receptor interference and whether this interference would select for resistant viruses with altered receptor usage. Chicken cells expressing the secreted ALV(A) SU immunoadhesin SU(A)-rIgG, which contains the subgroup A SU domain fused to the constant region of a rabbit immunoglobulin (IgG) heavy chain, showed significant receptor interference. A variant virus resistant to SU(A)-rIgG receptor interference was obtained. This virus had a six-amino-acid deletion in the subgroup A hr1 that altered receptor usage. This approach may identify regions of SU that play a critical role in receptor specificity.

INTRODUCTION

Retroviruses efficiently infect only those cells that express a specific receptor that can interact with the viral envelope glycoproteins (Hunter, 1997). Resistance to retrovirus infection can occur at the cell surface in two ways: (i) genetic resistance, i.e., a version of the specific receptor that can be recognized by the virus is not present on the cell surface; and (ii) receptor interference, i.e., the receptors are unavailable to retroviruses of the same subgroup due to interaction with the viral envelope glycoprotein in the secretory pathway and/or at the cell surface that leads to the degradation of the complex (Hunter, 1997; Weiss, 1982, 1992). The selective pressure of the host developing resistance to retroviral infection has been proposed as a major force in the evolution of retroviruses to alter their receptor usage (Coffin, 1990, 1992). The avian leukosis–sarcoma virus (ALV) group of retroviruses provides a powerful system for studying the envelope–receptor interactions involved in retrovirus entry because different members of this group of closely related viruses use distinct cellular receptors (Weiss, 1992). Analysis of the ALV envelope subgroups suggests that the different ALVs evolved from a common ancestor by mutations in the env gene.

ALVs have been divided into 10 envelope subgroups, A through J, based on host range, receptor interference patterns, and neutralization by antibodies (Weiss, 1992). The subgroup A to E viruses have been divided into noncytopathic (A, C, and E) and cytopathic (B and D) groups; cytopathic ALVs can cause a transient cytopathicity in 30–40% of the infected cells (Weller et al., 1980; Weller and Temin, 1981). Recently ALV replication in DF-1 cells, a permanent, nontransformed cell line derived from Line 0 chicken embryo fibroblasts, was described (Himly et al., 1998; Schaefer-Klein et al., 1998). Unexpectedly, infection of DF-1 cells with subgroup C ALV caused a transient cytoxicity similar to the cytotoxicity observed in avian cell cultures (including DF-1) infected with subgroup B and D viruses. Three genetic loci determine the susceptibility of chicken cells to the subgroup A to E ALVs, tv-a, tv-b, and tv-c (Weiss, 1992). Susceptibility to subgroup A ALV infection is controlled by tv-a, susceptibility to subgroup C by tv-c, and susceptibility to subgroups B, D, and E by tv-b. The receptor for subgroup A ALV, Tva, contains sequences related to the ligand-binding region of low-density lipoprotein receptors (Bates et al., 1993, 1998; Young et al., 1993). The receptor for subgroups B and D ALVs, CAR1, and the receptor for subgroup E ALV, SEAR, are related to tumor necrosis factor receptors (Adkins et al., 1997; Brojatsch et al., 1996; Smith et al., 1998). The subgroup C receptor has not yet been identified.

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The mature ALV envelope glycoproteins are trimers composed of the surface glycoprotein (SU) covalently bound to the transmembrane glycoprotein (TM) (Hunter, 1997). The major domains that interact with the host cell receptor are contained in SU while TM anchors SU to the cell membrane and appears to be directly involved in the fusion of the viral and cellular membranes. The subgroup A to E envelope glycoproteins are homologous and highly related (average 85% identity) except for five small regions in SU (vr1, vr2, hr1, hr2, and vr3) (Bova et al., 1986, 1988; Dorner et al., 1985). The results of several studies characterizing viruses with recombinant env genes suggest that the principal interactions between SU and the receptor that determine which receptor is recognized involve the hr1 and hr2 regions (Dorner and Coffin, 1986; Tsichlis and Coffin, 1980). The vr3 region may play a role in receptor recognition but does not appear to contribute to binding specificity; vr1 and vr2 do not appear to be necessary for receptor specificity.

The selective pressure of host resistance has apparently caused ALV to use alternative receptors. To test this hypothesis, Taplitz and Coffin passaged a subgroup B virus, td-Pr-RSV-B, on a mixture of permissive chicken (C/E) and nonpermissive quail (QT6/BD) cells and selected for variant viruses able to replicate in cells that lacked the subgroup B receptor (Taplitz and Coffin, 1997). A variant virus with an extended host range was identified that had two amino acid changes, L155S and T156I, in the hr1 region of SU (see Fig. 1B; the first two residues of RAV-2 in the box). The variant virus was able to use both the chicken subgroup B receptor and the quail subgroup E receptor. These data showed that small changes in the ALV envelope gene could alter receptor usage to circumvent genetic resistance.

Cells and animals that express subgroup A ALV envelope glycoproteins are highly resistant to superinfection by ALV(A) (Crittenden et al., 1989; Federspiel et al., 1991; Salter and Crittenden, 1989, 1991). We tested whether expression of a soluble form of subgroup A SU would cause receptor interference and select for resistant viruses with altered receptor usage. Chicken cells expressing the secreted ALV(A) SU immunoadhesin SU(A)-rIgG, which contains the subgroup A SU domain fused to the constant region of a rabbit immunoglobulin (IgG) heavy chain (Zingler and Young, 1996), showed significant resistance to ALV(A) infection. A variant virus resistant to SU(A)-rIgG receptor interference that contained a six-amino-acid deletion in the subgroup A hr1 was selected; this deletion altered receptor usage.

**RESULTS**

**SU(A)-rIgG significantly inhibits ALV(A) infection**

Clonal cell lines expressing SU(A)-rIgG were produced by calcium phosphate transfection of TFANE/O/SU(A)-rIgG plasmid DNA into DF-1 cells. DF-1 cells do not contain endogenous sequences closely related to the ALV-based vectors (Astrin et al., 1979), eliminating possible recombinants between the vector and endogenous viruses. To determine the level of resistance to ALV(A) infection, the cell lines were challenged with

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**FIG. 1.** (A) Schematic representations of the ALV-based RCASBP replication-competent retroviral vector and the major domains of the envelope glycoproteins. The five regions of amino acid sequence variation (vr1, vr2, hr1, hr2, and vr3) identified by comparing the sequences of the surface glycoproteins (SU) of ALV subgroups A to E are also shown. (B) Comparison of the amino acid sequences of the two major SU variable domains, hr1 and hr2, of representative ALV envelope subgroups A to E. The sequences were aligned with the ClustalW multiple alignment program of MacVector 6.5. Amino acids identical to SR-A are denoted by (●); gaps in the alignment are denoted by (−). The region in subgroup A hr1 deleted in the variant virus identified in this study and the homologous regions in subgroup B to E are highlighted in a box.
TABLE 1
Relative Resistance of DF-1 Cell Lines Expressing SU(A)-rIgG to ALV(A) Infection

<table>
<thead>
<tr>
<th>Cell line</th>
<th>RCASBP(A)AP titer</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected DF-1</td>
<td>$4.5 \times 10^4$</td>
<td>—</td>
</tr>
<tr>
<td>TFANEO alone</td>
<td>$2.9 \times 10^4$</td>
<td>1.5</td>
</tr>
<tr>
<td>TF/SU(A)-4</td>
<td>$1.2 \times 10^4$</td>
<td>4090</td>
</tr>
<tr>
<td>TF/SU(A)-7</td>
<td>$2.2 \times 10^4$</td>
<td>2045</td>
</tr>
<tr>
<td>TF/SU(A)-9</td>
<td>$4.6 \times 10^4$</td>
<td>978</td>
</tr>
<tr>
<td>TF/SU(A)-19</td>
<td>$2.4 \times 10^4$</td>
<td>187,500</td>
</tr>
<tr>
<td>TF/SU(A)-23</td>
<td>$1.3 \times 10^4$</td>
<td>36,000</td>
</tr>
<tr>
<td>TF/SU(A)-30</td>
<td>$1.5 \times 10^4$</td>
<td>3000</td>
</tr>
</tbody>
</table>

$^a$ The resistance of cells to ALV(A) infection was determined by dividing the average titer obtained on the control uninfected DF-1 cells by the average titer obtained for each experimental cell line.

RCASBP(A)AP. A range of resistance to infection by RCASBP(A)AP ($\sim1,000–185,000$-fold) was produced by the different cell lines (Table 1). The TF/SU(A)-19 cell line produced the highest level of resistance to infection by RCASBP(A)AP, $\sim185,000$-fold, and the antiviral effect was specific for ALV(A) (Table 2), which is consistent with a receptor interference mechanism.

Selection of RCASBP(A) variants resistant to the SU(A)-rIgG antiviral effect

TF/SU(A)-19 cells ($3.5 \times 10^6$) were challenged with RCASBP(A)AP (titer $5.3 \pm 1.9 \times 10^4$ ifu/ml) at a multiplicity of infection of 1.5, 3.0, or 7.6 ifu/cell. As a control, a cell line transfected with the TFANEO plasmid alone was challenged with either $\sim1$ or $\sim10$ ifu of RCASBP(A)AP to monitor the rate of virus spread in the absence of SU(A)-rIgG when the number of cells initially infected was low. The infected cell lines were passaged and monitored for virus production by assaying for the ALV CA protein by ELISA. ALV CA protein levels rose rapidly in the control cultures and reached peak levels 8–12 days postinfection (Fig. 2A). In the infected TF/SU(A)-19 cultures virus replication was seen only after infection at 7.6 ifu/cell; detectable levels of ALV CA were produced 20 days postinfection and peak levels were seen by day 32 (Fig. 2A). We observed a transient cytotoxicity in the infected TF/SU(A)-19 culture 32 days postinfection which lasted several days. The observed cytotoxicity was similar to the transient cytotoxicity previously observed in DF-1 cultures infected with either RCASBP(B) or RCASBP(C) viruses. All cultures were negative for subgroup B and C ALVs as assayed by PCR with subgroup-specific primers (data not shown).

To determine whether we had selected a viral population that replicates more efficiently in TF/SU(A)-19 cells than wild-type RCASBP(A)AP, uninfected TF/SU(A)-19 cells were challenged with 5 ml of day-36 supernatant from either the infected TF/SU(A)-19 culture or a control culture. The cultures were passaged and monitored for virus production by ELISA. The virus produced by the TF/SU(A)-19 culture (Fig. 2A) replicated better than wild-type RCASBP(A)AP in TF/SU(A)-19 cells (Fig. 2B).

The selected viruses contain a six-amino-acid deletion within the hr1 region of SU

The 11-kb SU region of the env gene was amplified by PCR from genomic DNA isolated from the day-20 TF/SU(A)-19 culture (Fig. 2B). The SU regions were amplified in two separate reactions, one using Taq DNA polymerase and one using Vent polymerase to control for any changes that might have been introduced by the PCR. The amplified products were cloned and the nucleotide sequence of SU was compiled from 22 clones. The deduced amino acid sequence of each clone was compared to the SU sequence of the parental virus RCASBP(A). All of the SU regions cloned from the TF/SU(A)-19 culture contained a deletion of amino acids 155–160 ($\Delta155–160$) in hr1 (Fig. 3). Several clones contained additional amino acid differences in SU compared to RCASBP(A).

A RCASBP(A)AP molecular clone with the SU $\Delta155–160$ mutation is resistant to the antiviral effect of SU(A)-rIgG

To determine whether the $\Delta155–160$ mutation provided a growth advantage in cells expressing SU(A)-rIgG, we replaced the SU fragment in the wild-type RCASBP(A)AP molecular clone with the SU fragment containing the $\Delta155–160$ mutation. Wild-type and mutant RCASBP(A)AP plasmid DNAs were transfected into both DF-1 and TF/SU(A)-19 cells. To determine the rate of viral spread in each culture, the culture supernatants were assayed for infectious virus on DF-1 cells by AP assay. The $\Delta155–160$ mutant virus produced infectious virus in DF-1 cells at a somewhat slower rate and to a 10- to 20-fold lower titer compared to wild-type RCASBP(A)AP (Fig. 4). In addition, a transient period of cytotoxicity was observed 12 to 16 days posttransfection in the $\Delta155–160$ virus-infected

TABLE 2
Relative Resistance of TF/SU(A)-19 Cells to ALV Infection

<table>
<thead>
<tr>
<th>Virus</th>
<th>DF-1 (mean ± SD)</th>
<th>TF/SU(A)-19 (mean ± SD)</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCASBP(A)AP</td>
<td>$5.3 \pm 1.9 \times 10^4$</td>
<td>$2.9 \pm 3.2 \times 10^4$</td>
<td>182,700</td>
</tr>
<tr>
<td>RCASBP(B)AP</td>
<td>$1.4 \pm 0.1 \times 10^4$</td>
<td>$1.0 \pm 0.5 \times 10^4$</td>
<td>1.4</td>
</tr>
<tr>
<td>RCASBP(C)AP</td>
<td>$3.0 \pm 1.3 \times 10^4$</td>
<td>$8.8 \pm 1.5 \times 10^4$</td>
<td>3.4</td>
</tr>
</tbody>
</table>

$^a$ The resistance of the TF/SU(A)-19 cells to virus infection was determined by dividing the mean titer obtained in the DF-1 cells by the mean titer obtained for each virus in TF/SU(A)-19 cells.
DF-1 culture, after which the cells recovered. The Δ155–160 mutant virus did not replicate as well in TF/SU(A)-19 cells as in DF-1 cells; the titer in TF/SU(A)-19 cells was 5- to 10-fold lower (Fig. 4). A transient period of cytotoxicity was also observed 24 to 28 days posttransfection in the Δ155–160 virus infected TF/SU(A)-19 cells. The TF/

![Graph](image1)

**FIG. 2.** Selection of variant subgroup A ALVs resistant to SU(A)-rIgG receptor interference. (A) TF/SU(A)-19 cells, a cell line derived from DF-1 cells transfected with the TFANEO/SU(A)-rIgG plasmid and expressing SU(A)-rIgG protein, were challenged with RCASBP(A)AP at a multiplicity of infection of 7.6 (○), 3.0 (□), or 1.5 (□) ifu/cell. As controls, the TFANEO control DF-1 cell line was challenged with ~1 ifu (●) or ~10 ifu (■) RCASBP(A)AP. Viral growth was monitored by ELISA for the ALV CA protein. The arrows indicate the period of transient cytotoxicity observed in the challenged TF/SU(A)-19 culture. (B) Uninfected TF/SU(A)-19 cells were infected with 5.0 ml of day-36 supernatant from the TF/SU(A)-19 culture (□) or from a control culture (■) (see A). Viral growth was monitored by ELISA.

![Graph](image2)

**FIG. 3.** Identification of a variant subgroup A virus. The SU regions of the env genes of ALV proviruses were cloned from genomic DNA isolated from the TF/SU(A)-19 infected culture. The genes were amplified by PCR and cloned as KpnI to SalI fragments, and the nucleotide sequence was determined. A schematic of the cloned region is included at the top. The amino acids are numbered from the start of the mature SU glycoprotein (gp85). The amino acids contained in the five variable regions are: vr1 64–75; vr2 100–105; hr1 122–165; hr2 199–227; and vr3 261–269. The deduced amino acid sequences of the hr1 and hr2 region of each SU clone compared to the wild-type sequence are shown. Clones 1–12 were amplified by Taq DNA polymerase; clones 13–22 were amplified by Vent DNA polymerase. The differences in amino acid sequence compared to SR-A SU (WT) are shown. Deletions in the alignment are denoted by (–). A stop codon is designated by (*). Any additional amino acid changes found in each clone are also listed.

![Graph](image3)
SU(A)-19 cells transfected with wild-type RCASBP(A)AP DNA failed to produce detectable levels of infectious wild-type virus (Fig. 4).

**Analysis of receptor interference patterns**

To determine whether the mutant Δ155–160 envelope glycoproteins altered the receptor usage of Δ155–160 RCASBP(A)AP, we performed a series of receptor interference assays. Uninfected DF-1 cells and DF-1 cells chronically infected with ALVs of subgroup A (RCASBP(A)), subgroup B (RCASBP(B)), subgroup C, (RCASBP(C)), or subgroup J (pHPRS103) were challenged with 10-fold serial dilutions of either the wild-type RCASBP(A)AP virus or the Δ155–160 mutant and the viral titer was determined by AP assay. Uninfected DF-1 cells and DF-1 cells infected with subgroups B, C, and J ALVs were efficiently infected by RCASBP(A)AP (Fig. 5). However, in DF-1 cells infected with subgroup A ALV, RCASBP(A)AP infection was inhibited by 5 logs (Fig. 5) and superinfection of subgroup B infected cells with RCASBP(B)AP or subgroup C infected cells with RCASBP(C)AP produced a 4-log decrease in titer (data not shown). The receptor interference profiles of the Δ155–160 RCASBP(A)AP mutant virus propagated in DF-1 cells and TF/SU(A)-19 cells were very similar (Fig. 5). The Δ155–160 mutant virus infected the subgroup A infected DF-1 cells much more efficiently (within 10-fold of the titer on DF-1 cells) compared to wild-type (Δ155–160) which may indicate that the Δ155–160 virus can use the Tvb and Tvc receptors to enter cells. The Δ155–160 mutant envelope glycoproteins can still interact with Tva.

To determine whether the deletion in the hr1 region in the Δ155–160 mutant resulted in a lower binding affinity for the Tva receptor, the Δ155–160 and wild-type

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**FIG. 4.** Replication of recombinant RCASBP(A)AP containing the Δ155–160 mutation. Plasmid DNAs containing the wild-type RCASBP(A)AP virus (open symbols) or a recombinant RCASBP(A)AP containing the Δ155–160 mutation (closed symbols) in proviral form were transfected into DF-1 (□ or ■) or TF/SU(A)-19 (○ or ●). Virus growth was monitored by determining the infectious titer produced by each culture by AP assay. The arrows define the period of cytotoxicity observed in the DF-1 and TF/SU(A)-19 cultures.

**FIG. 5.** Analysis of the receptor interference patterns of the Δ155–160 mutant and wild-type RCASBP(A)AP viruses produced in DF-1 cells and the Δ155–160 mutant in TF/SU(A)-19 cells. Uninfected DF-1 cells (DF-1) and DF-1 cells chronically infected with RCASBP(A) (A), RCASBP(B) (B), RCASBP(C) (C), or subgroup J HPRS-103 (J) were infected with 10-fold serial dilutions of the culture supernatants collected 20 days after transfection and the titer was quantitated by AP assay. The results shown are an average of three different experiments.
Relative Resistance of TF/sTva-4 Cells to Infection by Δ155–160 Mutant and Wild-Type RCASBP(A)AP Viruses

<table>
<thead>
<tr>
<th>Virus (cell type)</th>
<th>Titer (mean ± SD)</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF-1</td>
<td>TF/sTva-4*</td>
</tr>
<tr>
<td>Wild-type (DF-1)</td>
<td>5.3 ± 1.9 x 10⁶</td>
<td>3.4 ± 1.2 x 10⁶</td>
</tr>
<tr>
<td>Δ155–160 (DF-1)</td>
<td>2.5 ± 0.4 x 10⁶</td>
<td>4.5 ± 0.2 x 10⁶</td>
</tr>
<tr>
<td>Δ155–160 (TF/SUA-19)</td>
<td>4.4 ± 1.3 x 10⁶</td>
<td>7.9 ± 1.0 x 10⁶</td>
</tr>
</tbody>
</table>

* Virions were preabsorbed with supernatant from a confluent TF/sTva-4 culture containing the sTva-mIgG protein prior to assay.
* The resistance of the TF/sTva-4 cells to virus infection was determined by dividing the mean titer obtained in the DF-1 cells by the mean titer obtained for each virus in TF/sTva-4 cells. The fold difference in the resistance of the mutant virus compared to wild-type virus is given in parentheses.
efficiently infect cells. The deletion of six amino acid residues in the subgroup A hr1, the region of the subgroup B hr1 domain homologous to the region of the subgroup A hr1 (Fig. 1B; see box). These data may indicate that this region of hr1 plays a critical role in receptor specificity.

The deletion of residues 155–160 of subgroup A hr1 may have changed the structure of the surface glycoprotein such that virions produced with the Δ155–160 mutant envelope glycoproteins may be capable of infecting cells by interacting with other cell surface protein(s) in addition to Tva. Since the different ALV envelope subgroups may have evolved from a common ancestor because of host resistance, one possibility is that the Δ155–160 mutant can also use a different ALV receptor. We do not have conclusive data; however, the Δ155–160 mutant RCASBP(A)AP, RCASBP(B)AP, and RCASBP(C)AP viruses all replicate to similar titers and induce cytotoxicity in DF-1 cells. In addition, the receptor interference analysis showed that the Δ155–160 virus did not infect cells already infected with subgroup B or C ALV as efficiently as wild-type. Taken together, the Δ155–160 virus may use the Tvb and/or Tvc receptors, at least in part, to infect DF-1 cells. However, the Δ155–160 RCASBP(A)AP mutant virus did not show an altered receptor interference pattern for the subgroup J ALV receptor suggesting that it does not recognize this receptor.

These data suggest that the subgroup A to E ALVs may share a common link in virus entry. A link between the Tva, Tvb, and Tvc receptors, which excludes the subgroup J receptor, could explain the observation that the subgroup A to E ALV envelope glycoproteins are homologous and highly related, while the subgroup J ALV envelope glycoprotein is very different (e.g., ~44% identity with subgroup A). A possible link between these receptors could be explained by a common structural motif that is shared by these apparently very different proteins. It should be kept in mind that the Tva and Tvb receptors show no sequence relatedness and have no known common structural features. Another possible link could be a common accessory protein or coreceptor that is required for efficient virus entry. This would be similar to the situation in the entry of HIV-1 into susceptible cells. In this model, viral envelopes that recognize Tva, Tvb, and Tvc would also recognize the same or homologous coreceptor while a different coreceptor would be required for efficient ALV(J) infection. If this idea is correct, the putative coreceptor must be evolutionarily conserved and must also be expressed in a variety of species since the expression of the Tva and Tvb receptors in mammalian cells confers susceptibility to ALV infection.

MATERIALS AND METHODS

Envelope glycoprotein sequence alignment

The deduced amino acid sequences of the SU regions of subgroup A to E ALVs were compared using the ClustalW multiple sequence alignment program of MacVector 6.5 (Oxford Molecular Ltd., Oxford, England). The Schmidt-Ruppin subgroup A strain of Rous sarcoma virus (SR-A, GenBank Accession No. M14901), Prague subgroup C strain of Rous sarcoma virus (PR-C, GenBank Accession No. J02342), Rous associated virus type 2 (RAV-2, GenBank Accession No. M14902), Schmidt-Ruppin subgroup D strain of Rous sarcoma virus (SR-D,
GenBank Accession No. D10652), and Rous associated virus type 0 (RAV-0, GenBank Accession No. M12171) were used in the sequence alignments.

Vector constructions

The construction of the SU(A)-rIgG gene contained in the plasmid pKZ387 was described previously (Zingler and Young, 1996). The SU(A)-rIgG gene encodes the first 338 amino acids of the SR-A env gene fused to amino acids 96 to 323 of the constant region of the rabbit immunoglobulin G gene (GenBank Accession No. K00752). The SU(A)-rIgG coding region was subcloned into the NcoI and PstI sites of the adaptor plasmid CLA12NCO (Federspiel and Hughes, 1997). The SU(A)-rIgG gene was isolated as a Clal fragment from CLA12NCO and subcloned into the TFA NEO expression plasmid [TFANEO/SU(A)-rIgG]. The TFA NEO expression cassette consists of two LTRs derived from the RCAS vector that provide a strong promoter, enhancer, and polyadenylation sites flanking a unique Clal insertion site (Federspiel et al., 1989). The TFA NEO plasmid also contains a neo resistance gene expressed under the control of the chicken β-actin promoter and an ampicillin resistance gene for selection in Escherichia coli.

The construction of the RCASBP(A)AP retroviral vector, an ALV-based replication competent vector with a subgroup A env gene, and the heat stable human placental alkaline phosphatase gene (AP) has been described (Federspiel and Hughes, 1997; Fekete and Cepko, 1993; Fields-Berry et al., 1992). The SalI sites flanking the AP gene were made blunt with the Klenow fragment of DNA polymerase type I (New England Biolabs, Beverly, MA), and the modified fragment was cloned into the SalI site (also made blunt) of the CLA12 adaptor plasmid. The APsal gene was subcloned into the RCASBP(A) vector as a Clal fragment to produce RCASBP(A)APsal. The region encoding the Δ155–160 mutant SU was isolated on an Asp718 to SalI fragment from the pBluescript KS clone 20 (see Fig. 3) and cloned into the unique Asp718 and SalI sites of the RCASBP(A)APsal vector. The mutation in the env gene of the recombinant RCASBP(A)APsal clones was verified by nucleotide sequence analysis.

Cell culture and virus propagation

DF-1 cells (Himly et al., 1998; Schaefer-Klein et al., 1998) and QT6 cells (Moscovici et al., 1977) were grown in Dulbecco’s modified Eagle’s medium (Gibco/BRL) supplemented with 10% fetal bovine serum (Gibco/BRL), 100 units of penicillin/ml, and 100 μg of streptomycin/ml (Quality Biological, Inc, Gaithersburg, MD) at 39°C and 5% CO2. The generation of the 3T3pg950 cell line, a NIH3T3 cell line expressing the quai pg950 Tva receptor, was previously described (Gilbert et al., 1994). The 3T3pg950 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Gibco/BRL), 250 μg of G418/ml (Gibco/BRL), 100 units of penicillin/ml, and 100 μg of streptomycin/ml at 37°C and 5% CO2. The cultures were passaged 1:3 or 1:6 when confluent.

Virus propagation was initiated either by transfection of plasmid DNA that contained the retroviral vector in proviral form (Federspiel and Hughes, 1997) or by direct infection. In standard transfections, 5 μg of purified plasmid DNA was introduced into DF-1 cells by the calcium phosphate precipitation method (Kingston et al., 1989). Viral spread was monitored by assaying culture supernatants for ALV capsid protein (CA) by ELISA (Smith et al., 1979). Virus stocks were generated from cell supernatants. The supernatants were cleared of cellular debris by centrifugation at 2000g for 10 min at 4°C and stored in aliquots at −80°C. DF-1 cells transfected with the TFA NEO plasmid were grown in 500 μg G418/ml to select for neomycin-resistant cells. Clones were isolated using cloning cylinders (Bellco Glass Inc., Vineland, NJ), expanded, and maintained with standard medium supplemented with 250 μg/ml G418.

DF-1 cell cultures were chronically infected with RCASBP(A), RCASBP(B), RCASBP(C), or HPRS-103. The RCASBP viruses with subgroup (A), (B), and (C) env genes have been described previously (Federspiel and Hughes, 1997). HPRS-103 (GenBank Accession No. Z46390) is an ALV with a subgroup I env gene (Bai et al., 1995) and was obtained from Michael A. Skinner (Institute for Animal Health, Compton, Near Newbury, Berkshire, UK).

ELISA

The ALV CA protein was detected in culture supernatants by ELISA as previously described (Smith et al., 1979). The level of sTva-mlG was quantitated in culture supernatants by ELISA for the mouse IgG tag as previously described (Holmen et al., 1999).

Cloning and nucleotide sequence analysis of integrated viral DNA

DNA was isolated from cells in culture using the QIAamp Tissue Kit (Qiagen). The SU gene was amplified by PCR using either Taq DNA polymerase (Promega, Madison, WI) or Vent DNA polymerase (New England Biolabs). The PCR reactions were heated to 90°C for 1 min and initiated by the addition of 1.5 μl of Taq DNA poly-
merase diluted 1:10 v/v (0.75 units). Thirty cycles of PCR were carried out as follows: 90°C for 40 s, then 59°C for 80 s. Each Vent PCR contained 1.25 μl 10× Vent Pol buffer and 0.125 μl of 100× acetylated BSA (supplied by the manufacturer), 1.0 μl of each dNTP at 25 mM, 0.5 μl of each primer (A260 = 5), 0.625 μl of 0.1 M MgSO₄, 6.0 μl H₂O, 1.0 μl 50% DMSO, and 1.0 μl of DNA (genomic DNA ~100 ng/μl; plasmid DNA ~2 ng/μl). The reactions were heated to 90°C for 1 min and initiated by the addition of 0.5 μl of Vent DNA polymerase diluted 1:2 v/v (0.5 units). Thirty cycles of PCR were carried out as follows: 90°C for 40 s, then 59°C for 80 s.

The amplified products were separated by agarose gel electrophoresis and the 11-kb product was purified and digested with Asp718 and SalI. The digested product was cloned into pBluescript KS (Stratagene, LaJolla, CA) digested with Asp718 and SalI. The nucleotide sequence of the SU region was determined by the Mayo Clinic Molecular Biology Core on a Perkin–Elmer ABI PRISM 377 DNA sequencer (with XL upgrade) with PE Applied Biosystems ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit and AmpliTaq DNA polymerase (PE Applied Biosystems, Foster City, CA).

**ALV alkaline phosphatase challenge assay**

DF-1 cell cultures (~30% confluent) were incubated with 10-fold serial dilutions of the RCASBP/AP virus stocks for 36–48 h at 38°C. In a preabsorption AP challenge assay, the 10-fold viral serial dilutions were first mixed with 2 ml of supernatant containing sTva-mIgG for 3 h at 4°C and then assayed as above. The assay for alkaline phosphatase activity was described previously (Holmen et al., 1999). The RCASBP(A)AP, RCASBP(B)AP, and RCASBP(C)AP retroviral vectors which contain the AP gene have been described previously (Federspiel and Hughes, 1997).

**Fluorescence-activated cell sorting (FACS) analysis of envelope glycoprotein binding to sTva-mIgG**

Uninfected DF-1 cells or DF-1 cells infected with either wild-type or Δ155–160 mutant RCASBP(A)AP virus were removed from culture dishes with Trypsin de Larco (Quality Biological, Inc.) and washed with Dulbecco’s phosphate-buffered saline. The cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. Approximately 1 × 10⁶ cells in PBS supplemented with 1% calf serum (PBS-CS) were incubated with supernatant containing sTva-mIgG (1 ml total volume) on ice for 30 min. The cells were then washed with PBS-CS and incubated with 5 μl of sheep anti-mouse Ig linked to fluorescein (Amersham Pharma Biotech, Arlington Heights, IL) in PBS-CS (1 ml total volume) on ice for 30 min. The cells/sTva-mIgG/Ig-fluorescein complexes were washed with PBS-CS, resuspended in 0.5 ml PBS-CS, and analyzed with a Becton–Dickinson FACScalibur using CELLQuest 3.1 software.

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**REFERENCES**


