The Receptor for the Subgroup C Avian Sarcoma and Leukosis Viruses, Tvc, Is Related to Mammalian Butyrophilins, Members of the Immunoglobulin Superfamily

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The five highly related envelope subgroups of the avian sarcoma and leukemia viruses (ASLVs), subgroup A [ASLV(A)] to ASLV(E), are thought to have evolved from an ancestral envelope glycoprotein yet utilize different cellular proteins as receptors. Alleles encoding the subgroup A ASLV receptors (Tva), members of the low-density lipoprotein receptor family, and the subgroup B, D, and E ASLV receptors (Tvb), members of the tumor necrosis factor receptor family, have been identified and cloned. However, alleles encoding the subgroup C ASLV receptors (Tvc) have not been cloned. Previously, we established a genetic linkage between tve and several other nearby genetic markers on chicken chromosome 28, including tva. In this study, we used this information to clone the tve gene and identify the Tvc receptor. A bacterial artificial chromosome containing a portion of chicken chromosome 28 that conferred susceptibility to ASLV(C) infection was identified. The tve gene was identified on this genomic DNA fragment and encodes a 488-amino-acid protein most closely related to mammalian butyrophilins, members of the immunoglobulin protein family. We subsequently cloned cDNAs encoding Tvc that confer susceptibility to infection by subgroup C viruses in chicken cells resistant to ASLV(C) infection and in mammalian cells that do not normally express functional ASLV receptors. In addition, normally susceptible chicken DT40 cells were resistant to ASLV(C) infection after both tve alleles were disrupted by homologous recombination. Tvc binds the ASLV(C) envelope glycoproteins with low-nanomolar affinity, an affinity similar to that of binding of Tva and Tvb with their respective envelope glycoproteins. We have also identified a mutation in the tve gene in line L15 chickens that explains why this line is resistant to ASLV(C) infection.

Retroviruses require an interaction between the viral glycoproteins and a specific cell surface protein (receptor) to initiate entry into a cell (reviewed in references 32 and 56). The envelope glycoproteins of retroviruses are composed of trimers of two glycoproteins: the surface glycoprotein (SU), which contains the domains responsible for interaction with the host receptor, and the transmembrane glycoprotein (TM), which anchors SU to the membrane and mediates fusion of the viral and host membranes. The interaction of the SU glycoprotein with the host receptor usually involves multiple, noncontiguous determinants in both proteins that specify receptor choice and binding affinity and trigger a conformational change in the envelope glycoproteins that initiates the fusion process. Despite the complexity and specificity of the interaction between the viral glycoproteins and host receptors, closely related retroviruses carry envelope glycoproteins with mutations that alter receptor usage. The natural selection of retroviral subgroups with altered receptor usage may help the virus overcome host resistance and promote co-infection and may lead to heterotransmission.

The five highly related envelope subgroups of the avian sarcoma and leukemia viruses (ASLVs), subgroup A [ASLV(A)] to ASLV(E), are thought to be an example of the evolution of receptor usage by an ancestral retrovirus (reviewed in references 6 and 54). The ASLV(A) to ASLV(E) SU glycoproteins are almost identical except for five hypervariable regions, vr1, vr2, hr1, hr2, and vr3 (12, 13, 20, 21). Analyses of ASLV env genes have identified the hr1 and hr2 domains as the principal receptor interaction determinants; vr3 also plays a role in determining the specificity of receptor recognition (20, 21, 51, 52). In experiments that mimic the evolutionary forces of natural selection in cells resistant to ASLV entry, new viral variants with mutations in the ASLV envelope glycoproteins that altered receptor usage and binding affinity were selected; these mutations were in hr1 and vr3 (29, 30, 38, 42, 50).

ASLVs have been especially useful for studying the early events of retrovirus infection, not only because they have members with closely related SU glycoproteins that use different cellular receptors but also because several ASLV receptors...
have been cloned and soluble forms of these receptors have been developed. In chicken cells, three genetic loci determine the susceptibility and resistance to subgroup A to E ASLVs: tva [susceptibility to ASLV(A)], tvb [susceptibility to ASLV(B), ASLV(D), and ASLV(E)], and tvc [susceptibility to ASLV(C)] (53, 54). Susceptibility to ASLV infection is dominant, and therefore it is likely that the tva, tvb, and tvc ASLV resistance alleles contain defects that either block expression or alter the protein so that it is not an efficient ASLV receptor. The ASLV(A) receptors (Tva) are related to the family of low-density lipoprotein receptors (LDLR) (8, 57). Two tva genes have been cloned, from quail (8, 57) and chicken (22), that encode closely related Tva receptors but with critical differences in the conserved 40-amino-acid LDLR ligand binding domain important for ASLV(A) SU interaction (9, 30, 38, 45–47, 58, 59). Three highly related Tvb receptors have been identified and cloned, all of which are related to the tumor necrosis factor receptor (TNFR) family. There are two different tvb susceptibility alleles in chickens. The tvb\(^{a}\) allele confers susceptibility to subgroups B, D, and E; the tvb\(^{b}\) allele confers susceptibility only to subgroups B and D (1, 3). These alleles encode the chicken Tvb\(^{b}\) (3) and Tvb\(^{a}\) (14) receptors, respectively, and differ by a single amino acid change that presumably alters the structure of the Tvb\(^{a}\) protein so that it no longer functions as an ASLV(E) receptor. A third cloned tvb receptor, the turkey Tvb\(^{b}\) receptor (2), confers susceptibility to only subgroup E ASLV. A 15-amino-acid region of the chicken Tvb receptors, residues 32 to 46, can serve as a minimal receptor for ASLV(B) viruses. However, the Tvb determinants required for ASLV(B) viruses are different from those required for ASLV(A) viruses. A soluble form of the chicken Tvb\(^{b}\) receptor (svtb\(^{b}\)) has been expressed using baculovirus DNA constructs. The region containing the tvb gene was identified on this genomic DNA fragment and gene cassettes were isolated as ClaI fragments and subcloned into the RCASBP(A) vector (24). DF-1 cells were subsequently cloned into the EcoRI and BglII sites of the pS95 epidermal growth factor expression vector under the control of the simian virus 40 early promoter (25). Note that pTvc was derived from the tvc mRNA splice variant that lacks the last of the five small exons downstream from the transmembrane domain, amino acid residues 305 to 315 in Tvc. A similar cloning strategy was used to construct pTvc-F, an expression plasmid that contains the entire tvc DNA coding region. A gene encoding a soluble form of the chicken Tvc receptor (svtvc-mIgG) and a similar soluble form of the chicken Tvb receptor (svtb\(^{b}\)-mIgG) (1) was constructed as described previously for soluble forms of the chicken and quail Tva receptors (22, 30, 31). These genes encode the extracellular domain of the particular ASLV receptor fused to the constant region of a mouse immunoglobulin G (mIgG) heavy chain and are in the CLA12NCO adaptor plasmid (24). The stvb\(^{b}\)-mIgG and stvb\(^{b}\)-mIgG gene cassettes were isolated as ClaI fragments and subcloned into the ClaI site of the RCASBP(A) vector. DF-1 cells were infected with each virus, and infected cell supernatants that contained either the Tvc-mIgG protein or the Tvb\(^{b}\)-mIgG protein were collected. The chicken stvA-mIgG protein was collected in supernatants from a stable DF-1 cell line expressing the stvAmIgG gene in the TFANEO expression vector (22). The supernatants were cleared by centrifugation at 2,000 \(\times g\) for 10 min at 4°C and stored in aliquots at −80°C.

A gene encoding the SU glycoprotein from RCASBP(C) fused to the constant region of a rabbit immunoglobulin G (rIgG), SU(C)-rIgG, was constructed as described previously for SU(A)-rIgG (29) and SU(B)-rIgG (3). The SU(C)-rIgG gene was subcloned into the CLA12NCO adaptor plasmid, recovered as a ClaI fragment, and subcloned into the ClaI site of the RCASBP(A) vector (24). DF-1 cells were infected with RCASBP(A)SU(C)-rIgG virus, and supernatant from the infected cells containing the SU(C)-rIgG protein was collected. Supernatant containing the SU(A)-rIgG protein was collected from a stable DF-1 cell line expressing the SUA-rIgG gene in the TFANEO expression vector (29). The supernatants were cleared by centrifugation at 2,000 \(\times g\) for 10 min at 4°C and stored in aliquots at −80°C.

The replication-competent ASLV recombinant viruses RCASBP(A)AP, RCASBP(B)AP, and RCASBP(C)AP, containing the human heat-stable alkaline phosphatase (AP) gene, and RCASBP(A)GFP and RCASBP(C)GFP, containing the green fluorescent protein (GFP) gene, have been described previously. The replication-competent ASLV recombinant viruses RCASBP(A)AP, RCASBP(B)AP, and RCASBP(C)AP, containing the human heat-stable alkaline phosphatase (AP) gene, and RCASBP(A)GFP and RCASBP(C)GFP, containing the green fluorescent protein (GFP) gene, have been described previously.
usually (24). The receptor subgroup of the viral envelope glycoprotein is given in parentheses in these virus designations.

The rvc knockout constructs were generated by insertion of isogenic homology regions from the 5' and 3' ends of the DT40 rvc gene into the multiple cloning sites of the pLoxPuro and pLoxBsr vectors bearing the puromycin or blasticidin S resistance genes, respectively, driven by the chicken β-actin promoter (4). Homology region 1 was obtained as a PCR product from DT40 genomic DNA with primers KO1L and KO1R (Table 1), shortened at the 5' end by KpnI and at the 3' end by Sall digestion, and ligated into unique KpnI and Sall sites in the pLoxBsr vector. Homology region 1 is 1.907 bp in length and spans nucleotides −2923 to −1017 5' from the initiation ATG of rvc. Homology region 2 was obtained by PCR using primers KO2L and KO2R (Table 1), digested by SpeI in the noncomplementary part of the primers, and ligated into the unique SpeI site in the pLoxBsr vector. Homology region 2 is 2.788 bp in length and spans nucleotides 3033 to 5820 3' from the ATG. After the 5' and 3' target locus sequences were cloned into pLoxBsr, creating pLOXbsr, the blasticidin S resistance gene was replaced with the puromycin resistance gene from pLoxPuro by using the Sall and Xhol sites to create pLoxvcruro (4).

**ALV AP assay.** For AP assays, cells cultures (−30% confluent) were incubated with 10-fold serial dilutions of the appropriate RCASBP(AP) virus stocks for 36 to 48 h. The assay for alkaline phosphatase activity was described previously (31).

**BACs.** BACs containing chicken genomic DNA inserts were obtained from BACPAC Resources (Oakland, Calif.), from the Wageningen University Chick-FPC collection (http://www.zod.wau.nl/vf), and from Jerry Doggen (Michigan State University, East Lansing). A human BAC containing the GFP gene that was used as an experimental control was a gift from Matt Cotten (Axxima State University, East Lansing). A human BAC containing the GFP gene that was used in the present study was a gift from Matt Cotten (Axxima State University, East Lansing). A human BAC containing the GFP gene that was used as an experimental control was a gift from Matt Cotten (Axxima State University, East Lansing).

**Reverse transcription-PCR (RT-PCR) and PCR.** The following oligonucleotide primers were used in this study (all oligonucleotides are written 5' to 3')

TVC1, CTGACCGTTGCGCGGCAGGCTG; TVC2, TGGGATCTCCCTCTGTGGT; TVC3, CATTGGCGCCCGGCTCCG; TVC4, GTGCTGCTCCCTCAATAGTGG; TVC5, GGGTCGCTCTGGATTATGAC; TVC6, aagaattctctcaagttttttgtgcctgt; TVC7, CTGGTGTGGGAGGCAAGGCAC; TVC8, AGCCGTTGTTGGTATGCG; TVA1, CATGTGCTGCTGCTGCTGCTG; TVA2, GATGCAGGGGGCCCTGGGG; TVC3, CCTGGTGCTCCCCAATGCTG; TVC8, AGCCGTTGTTGGTATGCG; TVA1, CATGTGCTGCTGCTGCTGCTG; TVA2, GATGCAGGGGGCCCTGGGG.

PCR products were sampled after different numbers of cycles to determine the exponential phase of the reactions. To be within the exponential phase of the reactions, 30 amplification cycles were used to generate receptor-specific PCR products, and 24 amplification cycles were used to amplify the highly expressed gpdh transcripts in individual samples. The primer combinations and annealing temperatures used for this analysis were as follows: for amplification of rvc cDNAs, primers TVC6 and TVC4 and a truncated form of 56°C. The truncated form of TVC6 was amplified using primers TVC7 and TVC8 located in the 5' and 3' untranslated region sequences (Tn = 56°C). The truncated form of TVC6 was amplified using primers TVC7 and TVC8 located in the 5' and 3' untranslated region sequences (Tn = 56°C).

To compare the expression levels of rva, rhb, and rvc transcripts in various chicken tissues, the cDNA samples were amplified with specific primers by using Taq DNA polymerase (Takara, Kyoto, Japan) in the presence of 1% dimethyl sulfoxide and 1 M betaine. PCR amplifications were performed using the following conditions: 2 min at 94°C; 30 cycles of 15 s at 94°C, annealing for 40 s at primer-specific temperatures (see below), and 2 min at 68°C; and a final extension of 7 min at 68°C. The PCR products were quantitated 2 days after infection as described above. A human BAC containing the GFP gene that was used as an experimental control was a gift from Matt Cotten (Axxima State University, East Lansing). A human BAC containing the GFP gene that was used in the present study was a gift from Matt Cotten (Axxima State University, East Lansing).

**Assays for rvc receptor function.** Line L15 CE6s were seeded at 3 × 10^4 per 30-mm dish. The next day, the cells were transiently transfected using Lipofectamine 2000 with 2 μg of DNA. The DNA samples tested included different BAC clones, plasmid constructs containing DNA subcloned from BACs, the pRve plasmid, and DNA from control BAC or plasmid clones. Two days after transfection, the cells were infected with RCASBP(C)GFP at a multiplicity of infection of 4. The number of GFP-positive cells was determined 2 days later by fluorescence-activated cell sorting (FACS) with a Coulter Epics Elite ESP apparatus (Coulter Corporation, Hialeah, Florida) and analyzed with WinMDI software (J. Trotter, The Scripps Research Institute, San Diego, Calif.). For receptor analysis in stably transfected hamster cells, the NIL-2 cell line was transfected using Lipofectamine 2000 with 2 μg of plasmid pTva or pTvc together with 0.2 μg of plasmid pMCI NEO pola(Y) (Stratagene, La Jolla, Calif.), which contains the neomycin resistance gene. The transfected cells were grown for 10 days in G418 (500 μg/ml) to select for neomycin resistance. Cell clones were isolated from soft agar, expanded, and challenged with RCASBP(A)GFP and RCASBP(C)GFP at a multiplicity of infection of 4. The GFP-positive cells were quantitated 2 days after infection as described above.

**TABLE 1. Expression of Tvc confers susceptibility to ASLV(C) infection**

<table>
<thead>
<tr>
<th>Cells</th>
<th>ASLV receptor(s)</th>
<th>RCASBP(C)AP</th>
<th>RCASBP(A)AP</th>
<th>RCASBP(B)AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF-1</td>
<td>Tva, Tvb, Tvc</td>
<td>(2.4 ± 0.6) × 10^4</td>
<td>(4.5 ± 3.3) × 10^4</td>
<td>(4.2 ± 1.2) × 10^4</td>
</tr>
<tr>
<td>N1L-Tvc</td>
<td>Tvc</td>
<td>&lt;1</td>
<td>5.3 ± 2.5</td>
<td>3.0 ± 2.6</td>
</tr>
<tr>
<td>N1L-Tva</td>
<td>Tva</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>NIL</td>
<td>None</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Cells were infected with 10-fold serial dilutions of ASLV stocks of RCASBP(C)AP, RCASBP(A)AP, and RCASBP(B)AP viruses, and the titer was determined by the AP assay and presented as AP-positive foci per milliliter. The results are the averages and standard deviations from three experiments.

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Sequence prediction programs. The SignalP 3.0 program (10) was used to predict the location of the signal peptide cleavage site in the amino acid sequence of Tvc. The transmembrane region was predicted by using the TMHMM 2.0 server (http://www.cbs.dtu.dk/services/TMHMM). The IgV and B30.2 domains in Tvc were predicted by searching the Conserved Domain database with the BLAST program (37) or by performing the search against the Piam protein families database (7). The IgC domains in Tvc and other butyrophilins were not predicted by these domain prediction programs, in part because these domains exhibit considerable sequence variation. The presence and position of the IgC domain in Tvc were determined by performing a multiple-sequence alignment with butyrophilins from three species, which shows the conserved residues in IgC.

**Knockout of tvc in chicken DT40 cells.** To knock out one tvc allele, DT40 cells (5 × 10^5 in 400 μl of medium) were electroporated (25 μF and 700 V applied in a 4-mm cuvette) with 20 μg of linearized pLOXvBr by using the Gene Pulser Xcell (Bio-Rad). After electroporation, 5 × 10^3 cells were plated per microtiter well in 100 μl of growth medium. Twenty-four hours later the medium was changed to growth medium supplemented with 15 μg/ml blasticidin S (Infotogen). After 10 days of drug selection, drug-resistant colonies were cloned, expanded, and checked for homologous recombination. To knock out the second tvc allele, DT40 tvc^-/- cells were electroporated with 20 μg of linearized pLOXvBr to generate a puromycin-resistant clone for testing the sensitivity or resistance to homologous recombination of the pLOXvBr construct. Homologous recombination of pLOXvBr was detected using primer HR, complementary to nucleotides 4181 to 4159, and primer PR4 within the puromycin resistance gene sequence. Nontargeted tvc genes were detected using primer HR and primer PR4 from the Ig-like domain-coding sequence and complementary to nucleotides 460 to 479. The primers are listed in the “RT-PCR and PCR” section above. One DT40 tvc^-/- clone was used for testing the sensitivity or resistance to RCASBP(B)GFP and RCASBP(C)GFP viruses.

**ELISA.** The levels of the mgG fusion proteins were quantitated in culture supernatants by ELISA for the mouse IgG tag as previously described (31). The levels of the rIgG fusion proteins were quantitated in culture supernatants by ELISA for the rabbit IgG tag as previously described (29). The linear range for a standard experiment was between 0.5 and 50 ng of ImmunoPure IgG Fc per ml.

**Immunoprecipitations and Western immunoblot analysis.** The vtc-mgG, vTa-mgG, and vTvb3-mgG proteins were immunoprecipitated separately with anti-mouse IgG-agarose beads (Sigma) for ≥1 h at 4°C, and the SU(C)-mgG, SU(A)-mgG, and SU(B)-mgG proteins were immunoprecipitated separately with anti-rabbit IgG agarose beads (Sigma) for ≥1 h at 4°C. The protein-antibody agarose bead complexes were collected by centrifugation and washed twice in dilution buffer (50 mM Tris-buffered saline, 1% Triton X-100, 1 mg/ml bovine serum albumin), once in 50 mM Tris-buffered saline, and once in 0.05 M Tris-Cl, pH 6.8. The washed complexes were collected by centrifugation, resuspended in 50 μl Laemmli sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.0625 M Tris-Cl, pH 6.8, 0.1% bromophenol blue, 5% β-mercaptoethanol), and heated for 5 min at 100°C. The precipitated and denatured proteins were separated by SDS-12% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, blocked, and washed as described previously (31, 36). The immunoblots were probed with a either a peroxidase-conjugated goat anti-rabbit IgG (heavy plus light chains [H+L]) (50 ng/ml) or a peroxidase-conjugated goat anti-mouse IgG (H+L) (50 ng/ml) or a peroxidase-conjugated goat anti-rabbit IgG (H+L) (50 ng/ml) (Kirkegaard and Perry Laboratories, Gaithersburg, MD). After extensive final washing, immunodetection of the protein-antibody-peroxidase complexes was performed with the Western blot chemiluminescence reagent (DuPont NEN, Boston, MA). The immunoblots were then exposed to Kodak X-Omat film.

**Binding affinity analyzed by FACS.** DF-1 cells or DF-1 cells infected with either RCASBP(A), RCASBP(B), or RCASBP(C) were removed from culture with trypsin de Icaco (Quality Biological, Inc.) and washed with Dullbeco's phosphate-buffered saline (PBS). The cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min and then washed with PBS. Approximately 1 × 10^6 cells in PBS supplemented with 1% calf serum (PBS-CS) were incubated with supernatant containing one of the receptor-mgG or SU-IgG fusion proteins on ice for 30 min. The stable DF-1 cell lines TS/vTa-18 (expressing chicken sTva-mgG), TS/vTu-3S (expressing vTu-mgG), TS/vSu(A)-19 (expressing vSu(A)-mgG), and TS/vSu(B)-12 (expressing vSu(B)-mgG) were transfected with RCASBP(A)dsNvb-mgG-infected DF-1 cells (expressing sTvb-mgG); and RCASBP(AJsU(C)-mgG-infected DF-1 cells [expressing SU(C)-mgG] were the sources of the receptor-mgG or SU-IgG fusion proteins. The DF-1 cells were then washed with PBS-CS and incubated with either 5 μl of goat anti-mouse IgG (H+L) linked to phycoerythrin or 5 μl of goat anti-rabbit IgG (H+L) linked to phycoerythrin (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in PBS-CS (1 ml total volume) on ice for 30 min. The cell-soluble receptor-IgG-phycocyanin complexes were washed with PBS-CS, resuspended in 0.5 ml PBS-CS, and analyzed with a Becton Dickinson FACScan-lithium using CELLQuest software.

**KD calculations.** The maximum possible fluorescence and apparent dissociation constant (K_D) for each data set obtained from the FACS binding assays were estimated by fitting the data via nonlinear least squares to a log logistic growth curve function, f(y) = M[1 + e^(-log\(x\) - log\(K_D\))] (where y is the mean fluorescence, M is the maximum fluorescence, r is the rate, x is the concentration of the receptor-mgG or SU-IgG fusion protein, and K_D is the dissociation constant, which is defined as the concentration of the receptor-mgG or SU-IgG fusion protein at half-maximal binding (30).

**Nucleotide sequence accession number.** The complete nucleotide sequence of the nvc CDNA has been submitted to GenBank and assigned accession number AY847576.

**RESULTS**

Isolation of the tvc gene by positional cloning. Our previous genetic linkage analysis mapped the location of the nvc locus to within 1.1 centimorgans of the tva locus on chicken chromosome 28 (Fig. 1) (23). To clone the tvc gene, we obtained overlapping BAC genomic clones from this region of chromosome 28 from the Wageningen University ChickFPC collection (http://www.zod.wau.nl/vf) and from Jerry Dodgson (Michigan State University, East Lansing). Each BAC DNA was transiently transfected into line L15 CEES, which are resistant to ASLV(C) infection, and challenged with RCASBP(C)GFP, an ASLV(C) virus without the GFP gene (24, 48). The challenged culture was scored for infection by the RCASBP(C)GFP virus by fluorescence microscopy. The transfection
efficiency of BAC DNA was extremely low by either the lipofection or calcium phosphate precipitation methods (data not shown). The transfection efficiencies were estimated using a control BAC clone containing a 130-kb human DNA genomic insert and a GFP expression cassette (5) and averaged 20 GFP-positive cells out of 10^6 transfected CEFs. Despite the extremely low BAC DNA transfection efficiencies, there were GFP-positive cells observed in RCASBP(C)GFP-challenged line L15 CEF cultures transfected with BAC CH261-100I9 but not in those transfected with the other chicken BAC clones, including the adjacent BAC CH261-132C17 (data not shown). The draft sequence of the chicken genome from Washington University (http://pre.ensembl.org/Gallus_gallus) enabled the mapping of the BAC clones to their locations on chromosome 28. The BAC CH261-100I9 clone contains ~200 kb of chicken genomic chromosome 28 DNA (draft positions ~681000 to 883000) and the tva gene (~818000 to 819500).

We analyzed the ~90-kb chicken DNA insert region of the BAC CH261-100I9 between the tva gene and the overlap with the BAC CH261-132C17 insert. Fragments of this region were subcloned into a plasmid expression vector by using convenient restriction enzyme sites, and each fragment was screened for the ability to confer susceptibility to RCASBP(C)GFP infection by transient transfection into line L15 CEFs as described above. This analysis mapped the tve gene to a 15-kb fragment of chromosome 28 (positions 735137 to 750326 in the draft sequence) (data not shown). Further deletions narrowed the location of the tve gene to the 10-kb region between positions 740000 and 750000 (data not shown). The Ensemble browser (http://www.ensembl.org/) predicted only one gene in this region, encoding a homologue to mammalian butyrophilins.

**Cloning of cDNAs encoding the putative Tvc receptor.** A tentative nucleotide sequence of the putative tve coding region was assembled using the Ensemble gene prediction software and sequences in the chicken expressed sequence tag databases (11, 17). Using RT-PCR and primers based on the assembled tve sequence, a ~1,200-bp CDNA product was amplified from total RNA isolated from the ASLV(C)-susceptible line H6 CEFs (data not shown). To obtain a complete cDNA, additional primers were designed from the nucleotide sequence of this initial cDNA clone and were used to amplify the 5' end (5'-RACE) and 3' end of the tve CDNA (data not shown). The resulting sequences were assembled into a tve CDNA consensus sequence (Fig. 2). This tve CDNA consensus sequence was verified by PCR amplification of full-length tve cDNAs by using primers based on the ends of the consensus sequence (data not shown). The tve mRNA is 1,875 nucleotides long with a single open reading frame encoding 488 amino acids. The deduced amino acid sequence of Tvc was used in a protein-protein BLAST search for homologous protein sequences in the National Center for Biotechnology Information protein databases. Figure 3 compares the Tvc protein sequence to the two most similar proteins identified in this search, human and bovine butyrophilin BTN1A1 (butyrophilin, subfamily 1, member A1) and mouse butyrophilin BTN1A1.

The butyrophilins are members of the immunoglobulin superfamily and are type I, single-transmembrane proteins with several conserved features (18, 39, 43). The extracellular regions of most butyrophilin proteins contain two immunoglobulin-like domains, IgV and IgC, which are related to CD80 and CD86 costimulatory molecules of the immune system. The cytoplasmic domain contains a B30.2 domain, a domain present in a large number of proteins, that may function as a protein-binding domain. Some members of the butyrophilin family are highly expressed in secretory epithelium of the mammary gland during lactation; other butyrophilin homologues are expressed predominately in skeletal muscle, intestine, or erythroid cells; while still other homologues are widely expressed in many tissues. To date, the function of any of the butyrophilin proteins is not understood, but the conserved structural domains and diverse expression profiles suggest that these proteins may have important general and tissue-specific functions within and outside the immune system.

We used computer predictions and the homology between Tvc and the mammalian butyrophilins to identify the putative signal peptide, the IgV domain, the IgC domain, the transmembrane domain, and the B30.2 domain in the Tvc protein sequence and the 5' and 3' noncoding regions of the tve CDNA (Fig. 2). The extracellular region of Tvc contains 227 amino acids with two potential N-linked glycosylation sites and four cysteine residues, the transmembrane region is 24 amino acids long and the cytoplasmic region contains 215 amino acids. The exon boundaries of the tve gene were determined by comparing the tve CDNA nucleotide sequence to the draft chicken genome sequence (Fig. 2). All tve intron-exon boundaries are phase 1 junctions, the major domains are each encoded on a single exon, and the sequence between the transmembrane and B30.2 domains contains five short exons, all characteristics shared by other butyrophilin family genes.

**Expression of the tve CDNA confers susceptibility to ASLV(C) infection.** To test the ability of the tve CDNA to confer susceptibility to ASLV(C) infection, the expression plasmid pTvc was constructed from a fragment of the tve CDNA encoding the extracellular domain, transmembrane domain, and a truncated cytoplasmic domain of Tvc (Fig. 4A). Line L15 CEFs were transiently transfected with either pTvc DNA or a control plasmid DNA, pTva, encoding the extracellular domain, transmembrane domain, and a truncated cytoplasmic domain of the chicken Tva receptor (22), and subsequently challenged with RCASBP(C)GFP. The challenged cultures were analyzed by phase-contrast and fluorescence microscopy (Fig. 4B), and the GFP-positive infected cells were quantitated by flow cytometry (Fig. 4C and D). CEFs transfected with pTvc were efficiently infected by RCASBP(C)GFP; on average 15% of the cells were GFP positive. In contrast, CEFs transfected with the pTva control plasmid were not efficiently infected by RCASBP(C)GFP and yielded only rare GFP-positive cells (<0.05%). These experiments demonstrate that the expression of Tvc confers susceptibility to ASLV(C) infection in chicken cells normally resistant to ASLV(C) infection. We initiated these experiments with this truncated tve CDNA at a time when a cDNA encoding the complete B30.2 cytoplasmic domain was not yet cloned. This tve CDNA did contain the complete extracellular domain that is important for its function as a ASLV(C) receptor. We have since repeated these experiments with the expression plasmid pTvc-F, which contains the entire tve CDNA coding region, and obtained similar results (data not shown). In addition, no toxicity was observed in cells expressing either the full-length or truncated Tvc protein.
Mammalian cells do not normally express functional receptors for any of the ASLVs. To further characterize the specificity of ASLV susceptibility conferred by Tvc, stable mammalian cell lines that express either a truncated form of the Tvc receptor or the chicken Tva receptor were generated. Hamster NIL cells were transfected with either the pTvc or pTva plasmid and stable lines generated that express each receptor, named NIL-Tvc and NIL-Tva, respectively. NIL-Tvc cells and NIL-Tva cells were challenged with RCASBP(C)GFP or RCASBP(A)GFP (the same ASLV but with a subgroup A
The challenged cultures were analyzed by phase-contrast and fluorescence microscopy, and the GFP-positive cells were quantitated by flow cytometry. The NIL-Tvc cells were efficiently infected with RCASBP(C)GFP but not with RCASBP(A)GFP, while the NIL-Tva cells were efficiently infected with RCASBP(A)GFP but not with RCASBP(C)GFP (data not shown). The titers of RCASBP(C)AP, RCASBP(A)AP, and RCASBP(B)AP virus stocks on the NIL cell lines and chicken DF-1 cells were compared. As expected, DF-1 cells were efficiently infected by viruses of all three envelope subgroups, since DF-1 cells express Tvc, Tva, and Tvb receptors (Table 1). NIL-Tvc cells were efficiently infected only by RCASBP(C)AP, and NIL-Tva cells were efficiently infected only by RCASBP(A)AP. The parental NIL cells, which do not express functional ASLV receptors, were not efficiently infected by any of these ASLV viruses.

**Targeted deletion of tvc renders DT40 cells resistant to ASLV(C) infection.** The tvc gene was deleted in chicken DT40 cells, a B-cell line with high rates of homologous recombination (4, 16). The 5' and 3' genomic regions that flank the tvc gene in DT40 cells were cloned and used for homologous recombination to target integration to completely delete the tvc coding sequence (Fig. 5A). A DT40 tvc<sup>−/−</sup> cell clone with a cell morphology, viability, and growth rate similar to those of parental DT40 cells was chosen for further study. Parental DT40 cells and DT40 tvc<sup>−/−</sup> cells were challenged with RCASBP(C)GFP and RCASBP(B)GFP viruses and analyzed by phase-contrast and fluorescence microscopy (Fig. 5B). Parental DT40 cells were infected by both RCASBP(C)GFP (Fig. 5B, panel b) and RCASBP(B)GFP (Fig. 5B, panel f). In contrast, DT40 tvc<sup>−/−</sup> cells were highly resistant to RCASBP(C)GFP (Fig. 5B, panel d) but still susceptible to infection by
RCASBP(B)GFP (Fig. 5B, panel h). These data confirm that tvc is the ASLV(C) receptor.

Tvc and ASLV(C) glycoproteins bind with low-nanomolar affinity. Two approaches were used to estimate the binding affinities of ASLV receptors for ASLV envelope glycoproteins. In one approach, the ASLV envelope glycoproteins expressed on the surface of DF-1 cells infected with ASLV(C), ASLV(A), or ASLV(B) were assayed for their ability to bind soluble forms of three ASLV receptors, sTvc-mIgG, sTva-mIgG, and sTvbS3-mIgG, by FACS as described previously (22, 30, 38). In a second approach, the ASLV receptor proteins expressed by DF-1 cells were assayed for their ability to bind soluble forms of ASLV SU glycoproteins, SU(C)-rIgG, SU(A)-rIgG, and SU(B)-rIgG, by FACS. The integrity of the three soluble receptor proteins and the three soluble SU proteins was determined by immunoprecipitation and Western analysis (Fig. 6A). The concentration of each protein stock was quantitated by ELISA for either the mouse or rabbit IgG (29, 31).

For the soluble receptor protein concentrations assayed, sTvc-mIgG bound only to ASLV(C)-infected cells (Fig. 6B), sTva-mIgG bound only to ASLV(A)-infected cells (Fig. 6C), and sTvbS3-mIgG bound only to ASLV(B)-infected cells (Fig. 6D). All three soluble receptor forms bound their respective envelope glycoproteins with subnanomolar affinity (Table 2). The estimated binding affinity of the sTva-SU(A) interaction (0.05 nM) was 10-fold higher than the sTvc-SU(C) (0.55 nM) and sTvbS3-SU(B) (0.9 nM) binding affinities in these experiments. We were able to detect the binding of SU(C)-Tvc and SU(A)-Tva interaction, but not SU(B)-Tva interaction, to uninfected DF-1 cells (Fig. 6E). The binding affinities measured for the SU(C)-Tvc and SU(A)-Tva interaction (0.05 nM) were both 10-fold lower than those we obtained using the soluble receptor approach (Table 2).

The tvc gene in the ASLV(C)-resistant line L15 contains a mutation that introduces a premature stop codon. Line L15 is highly resistant to ASLV(C) infection, presumably due to a mutation in the tvc gene. To test this hypothesis, we cloned tvc cDNAs from line L15 total RNA by RT-PCR using primers that amplified the complete transcript. When the nucleotide sequences of the line L15 tvc cDNA and the line H6 tvc cDNA were compared, one nucleotide difference was found that changed codon 55 (TGC, cysteine) to a termination codon (TAA, stop). The tvc gene in the ASLV(C)-resistant line L15 contains a mutation that introduces a premature stop codon.
We are designating the chicken line L15 tvcr gene. There are now five different mutations identified in the subgroup A to E ASLV receptors that result in resistance to infection by specific ASLV envelope subgroups in inbred White Leghorn chickens (1, 2, 22, 34; this study). The molecular defects encoded by these mutations either alter the structure of the receptor and reduce the binding affinity to the ASLV glycoprotein (e.g., tvra and tvbS3) or eliminate the expression of the receptor (e.g., tvca, tvbca2, and tvb). These mechanisms are consistent with the recessive nature of the ASLV-resistant phenotypes.

**Distribution of the ASLV receptor transcripts in chicken tissues.** To characterize the distribution of Tvc, Tva, and Tvb receptors in chickens, the receptor mRNA expression levels were analyzed by semiquantitative RT-PCR. A fragment of each receptor transcript was amplified from total RNA isolated from a variety of tissues from outbred Brown Leghorn chickens susceptible to ASLV subgroups A, B, and C. The pattern of receptor mRNA expression differs for each receptor (Fig. 7). The tvc mRNA is preferentially expressed in thymus, spleen, and bursa, organs involved in immune function. The tva mRNA levels are more abundant in ovary and testes, while tvb mRNA is more broadly expressed. Although there are differences in receptor mRNA expression levels, tvc, tva, and tvb mRNAs can be detected in all tissues if the cycles of amplification are increased, with the exception that tva mRNA was not detected in breast muscle.

**DISCUSSION**

It is likely that the related subgroup A to E ASLV env genes evolved from a single ancestral gene. The ability to use different cellular proteins as receptors would help the virus counter the development of resistance and host receptor variation. Three cell surface proteins have been identified as receptors for the subgroup A to E ASLVs. Tvc, the receptor for sub-

![Fig. 6. Binding affinity of the ASLV envelope glycoproteins for ASLV receptors. (Panel A) Western immunoblot analysis of the soluble forms of the Tvc receptor sTvc-mIgG (sTvc) the chicken Tva receptor TvaSsTva-mIgG (sTva), and the Tvb receptor sTvbS3-mIgG (sTvb) immunoprecipitated with anti-mouse IgG-agarose beads and of the secreted forms of the SU glycoproteins SU(C)-rIgG (SUC), SU(A)-rIgG (SUA), and SU(B)-rIgG (SUB) immunoprecipitated with anti-rabbit IgG-agarose beads. The precipitated proteins were denatured, separated by SDS-12% polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The filters were probed with either peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG, and the bound protein-antibody complexes were visualized by chemiluminescence using Kodak X-Omat film. Molecular masses (in kilodaltons) are given on the left. (Panels B to E) DF-1 cells chronically infected with either ASLV(C) (panel B), ASLV(A) (panel C), or ASLV(B) (panel D) and uninfected DF-1 cells (panel E) were fixed with paraformaldehyde and incubated with different amounts of each soluble receptor (panels B to D) or each secreted SU-rIgG (panel E). The receptor-viral glycoprotein complexes were bound to either goat anti-mouse IgG or goat anti-rabbit IgG linked to phycoerythrin. The amount of phycoerythrin bound to the cells was determined by FACS, and the maximum fluorescence was estimated (see Materials and Methods). The data were plotted as percent maximum fluorescence bound versus soluble receptor sTvc-mIgG (C), sTva-mIgG (A), or sTvb-mIgG (B) concentration (panels B to D) or secreted SU(C)-rIgG (C), SU(A)-rIgG (A), or SU(B)-rIgG (B) concentration (panel E). The values shown are averages and standard deviations from three experiments.
The dual requirement is, as far as ASLVs likely require both receptor binding and low pH to yet been proven experimentally, all of the subgroup A to E fusion of the viral and cellular membranes. Although it has not of the proteins and carry out the multistep process leading to ASLV SU glycoproteins have evolved the ability to bind each nor do they appear to be functionally related. However, the proteins have no obvious sequence or structural homology, which may be important or required for the receptors to initiate the fusion process, which does not involve a low-pH step. Second, the Ig, LDLR, and TNFR protein families have one or more conserved extracellular domains that may be important for their interactions with the ASLV SU glycoproteins (e.g., the 40-amino-acid LDLR ligand binding domain in Tva and the three cysteine-rich domains in Tvb). These conserved protein domains, which appear to be very different, may present some as-yet-unidentified combination of receptor determinants that enables the evolution of ASLV SU/receptor usage. Testing of this hypothesis will require a more complete understanding of the structures of the env proteins and the receptors and their interactions. Third, each ASLV SU glycoprotein is a member of a family of homologous proteins with related functions. Retrovirus infection causes, in infected cells, the synthesis of viral envelope glycoproteins that can down-regulate and/or block the normal functions of the receptor. The related family members may compensate for functional loss of the ASLV receptor protein in infected cells. The normal functions of Tvc, Tva, and Tvb proteins in chickens are not known; however, viral infection presumably does not involve the normal function of the receptor, so a physiologically functional receptor protein is probably not required.

The levels of the endogenous Tva and Tvb receptors expressed on avian cells appear to be extremely low. Because the receptor levels are low and the immunological reagents that can be used to detect ASLV SU glycoproteins and their receptors are few, the binding affinity of the ASLV SU/receptor interactions has been estimated using IgG-tagged forms of SU and/or receptor. Using these approaches, we estimate that subgroup C, A, and B ASLV SU glycoproteins bind their respective receptors with low-nanomolar affinities (Fig. 6; Table 2). This affinity may be important for optimal infection efficiency. ASLV(A) variants carrying mutations in SU that significantly reduced the binding affinity for the quail Tva receptor infected quail cells inefficiently (30, 38). Using the SU-IgG reagents, we were able to detect endogenous Tva and Tvc on DF-1 cells, but not endogenous Tvb. The two experimental approaches used to measure ASLV SU receptor binding affin-

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<th>Cells</th>
<th>Receptor</th>
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<tr>
<td></td>
<td>sTvc-mIgG</td>
<td>sTva-mIgG</td>
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<tr>
<td>DF-1/ASLV(C)</td>
<td>0.55 ± 0.20</td>
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<td>DF-1/ASLV(B)</td>
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^a Apparent KD values were estimated by fitting the data via nonlinear least squares to a log logistic growth curve function as described in Materials and Methods. Each result is the average and standard deviation from three experiments. 
^b NDB, no detectable binding. 
^c —, binding reaction not performed.

While the Ig, LDLR, and TNFR protein families are not homologous, they share several basic characteristics. First, these proteins are all type I, single-transmembrane glycoproteins, which may be important or required for the receptors to interact with ASLV SU. Most other retroviral env proteins use receptors that have multiple membrane-spanning domains; the SUs interact with several of the extracellular loops of the receptor to initiate the fusion process, which does not involve a low-pH step. Second, the Ig, LDLR, and TNFR protein families have one or more conserved extracellular domains that may be important for their interactions with the ASLV SU glycoproteins (e.g., the 40-amino-acid LDLR ligand binding domain in Tva and the three cysteine-rich domains in Tvb). These conserved protein domains, which appear to be very different, may present some as-yet-unidentified combination of receptor determinants that enables the evolution of ASLV SU/receptor usage. Testing of this hypothesis will require a more complete understanding of the structures of the env proteins and the receptors and their interactions. The related family members may compensate for functional loss of the ASLV receptor protein in infected cells. The normal functions of Tvc, Tva, and Tvb proteins in chickens are not known; however, viral infection presumably does not involve the normal function of the receptor, so a physiologically functional receptor protein is probably not required.

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**FIG. 7.** RT-PCR amplification of tva, tvb, and tvc transcripts from various chicken tissues. The cDNA samples were prepared from various tissues of outbred Brown Leghorn chickens or from CEFs from inbred line M. PCR primers were designed to amplify short regions in the N-terminal regions of the tva, tvb, and tvc genes (see Materials and Methods); the lengths of the expected products are indicated on the left. The figure shows photographs of PCR products after 30 cycles of amplification, separated on agarose gels and stained with ethidium bromide. The 30 PCR cycles were within the exponential phase of product amplification. By increasing the number of amplification cycles, the receptor transcripts were detectable in all tissues (except for tva in breast muscle). GAPDH was included as a standard. Neg., no-cDNA PCR control.
ities produced slightly different estimates of the affinities of these protein-protein interactions (Table 2). This apparent discrepancy may not be surprising, since in each approach, an IgG fusion protein that likely forms a dimer is used as one component of the SU-receptor interaction. However, the natural SU-receptor interaction involves trimeric ASLV glycoproteins binding to monomeric receptors. Despite these caveats, we believe that the estimates of both the relative and the absolute binding affinities of ASLVs for their receptors are reasonable.

The tissue distribution of retroviral receptors may be a determinant of pathogenicity. The ability of ASLVs to efficiently infect cells that express the receptors at extremely low levels has made a systematic characterization of ASLV subgroup A and B tissue tropisms and their possible effect(s) on pathogenicity difficult. We were able to detect tvc, tva, and tvb mRNAs by RT-PCR in almost all the tissues we tested, although there were differences in the expression profiles (Fig. 7). This result supports previous reports that if the bird is genetically susceptible, most chicken tissues can be infected by all three ASLV subgroups (15, 44). If most or all chicken cells express low levels of all three ASLV receptors, the tissue tropism of virus infection could be influenced by the subtle differences in receptor level. However, tropism could also be affected by other factors (sites of integration, long terminal repeat structure, and promoter/enhancer specificity, for example), and receptor expression may not necessarily be the major factor determining tropism or pathology.

The genetic linkage of the tva gene and the tvc gene in the chicken genome is striking. This close genetic linkage made it possible to use a positional approach to clone Tvc. It had been suggested that because of this linkage that the Tvc receptor would be related to the Tva receptor and that the two loci would derive from gene duplication. However, as this study shows, Tvc and Tva belong to very different protein families. At present, the close proximity of the tvc and tva genes on chicken microchromosome 28 appears to be a fortuitous coincidence. The tvb gene is located on chicken microchromosome 22 (27). This strengthens the argument that the proximity of the tva and tvc loci on chromosome 28 is an evolutionary accident.

The availability of three distinct receptor-SU pairs makes the ASLV system useful for additional evolutionary studies and structure/function analyses. The fact that all three of the receptors are type I membrane proteins has made it possible to express soluble versions of the receptors; this will make it easier to study the interactions between the receptors and their cognate SU's biochemically and, we hope, structurally. The fact that the structural changes in the ASLV env proteins required to cause the fusion of the viral and cellular membranes involve both receptor binding and low pH should make it possible to dissect the molecular details of the events that lead to membrane fusion.

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