JOURNAL OF VIROLOGY, Aug. 2005, p. 10408–10419 Vol. 79, No. 16
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.16.10408–10419.2005

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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Received 2 February 2005/Accepted 9 May 2005

The five highly related envelope subgroups of the avian sarcoma and leukosis 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 *tvc* and several other nearby genetic markers on chicken chromosome 28, including *tva*. In this study, we used this information to clone the *tvc* 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 *tvc* 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 *tvc* 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 *tvc* 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 coinfection and may lead to heterotransmission.

The five highly related envelope subgroups of the avian sarcoma and leukosis 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

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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^r, tvb^r, and tvc^r 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^{s1} allele confers susceptibility to subgroups B, D, and E; the tvb^{s3} allele confers susceptibility only to subgroups B and D (1, 3). These alleles encode the chicken Tvb^{S1} (3) and Tvb^{S3} (14) receptors, respectively, and differ by a single amino acid change that presumably alters the structure of the Tvb^{S1} protein so that it no longer functions as an ASLV(E) receptor. A third cloned *tvb* receptor, the turkey Tvb^{T} 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(E) entry appear to be conformation dependent and require residues distinct from ASLV(B) (1, 3, 35).

To date, alleles encoding the ASLV(C) receptor, Tvc, have not been cloned. We have cloned the *tvc* gene and identified the Tvc receptor. Previously, two studies reported that the genetic loci controlling the susceptibility to subgroup A and subgroup C ASLVs are closely linked in chickens (40, 41). We refined the linkage of the *tva* and *tvc* genes and mapped these loci to a specific region of chicken chromosome 28 (23). This information allowed us to identify a bacterial artificial chromosome (BAC) containing a portion of chicken microchromosome 28 that conferred susceptibility to ASLV(C) infection. The region containing the *tvc* gene was located on a 15-kb fragment by using subclones constructed from this BAC. The *tvc* gene was identified on this genomic DNA fragment and encodes a 488-amino-acid protein related to mammalian butyrophilins, members of the immunoglobulin protein family (18, 39, 43).

MATERIALS AND METHODS

Chicken lines. The inbred White Leghorn chicken lines H6 and L15 were originally developed at the Northern Poultry Breeding Station (Reaseheath, Cheshire, United Kingdom) and imported to Prague, Czech Republic, in 1989 and 1977, respectively. Line H6 is sensitive and line L15 resistant to ASLV(C) infection (23). The inbred White Leghorn chicken line M was developed at the Institute of Molecular Genetics (Prague, Czech Republic) and is sensitive to ASLV(C) infection (26).

Cell culture and virus propagation. Chicken embryo fibroblasts (CEFs) were prepared from 10-day-old embryos of chicken lines H6, L15, M, and Brown Leghorn as described previously (24). CEFs and DF-1 cells, a continuous fibroblastic cell line derived from line 0 CEFs (28, 48), were grown in Dulbecco's modified Eagle's medium–nutrient mixture F-12 Ham (Sigma, St. Louis, Missouri) supplemented with 5% calf serum, 1% fetal calf serum, 1% chicken serum, 10% tryptoso-phosphate broth, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) in a 5% CO_2 atmosphere at 37°C. For some experiments, DF-1 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL), 100 units of penicillin per ml, and 100 μg of streptomycin per ml (Quality Biological, Inc., Gaithersburg, MD) at 39°C and 5% CO_2. The immortalized fibroblastoid Syrian hamster cell line NIL-2 (19) was cultivated in Dulbecco's modified Eagle's medium–nutrient mixture F-12 Ham supplemented with 5% calf serum and antibiotics under the same conditions as for CEFs. The B lymphoid chicken $DT40^{Cre1}$ cells (4, 16) and the $DT40tvc^{-/-}$ derivative were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% chicken serum, glutamine, antibiotics, and 0.1 mM β-mercaptoethanol in a 5% CO_2 atmosphere at 41°C. They were passaged by seeding 10^5 cells/ml every 3 to 4 days.

Two methods were used to generate the replication-competent ASLV long terminal repeats (LTR) with a splice acceptor, equipped with Bryan polymerase (RCASBP). In one method, 3×10^5 DF-1 cells were seeded per well in a 24-well multidish (Nunclon, Roskilde, Denmark) and transfected the next day with 1 μg of the RCASBP(A)GFP, RCASBP(B)GFP, or RCASBP(C)GFP plasmid (24) by using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif.) according to the manufacturer's procedure. Cells were subcultured until they reached confluence on 100-mm culture dishes. The supernatant was collected and centrifuged at $2,000 \times g$ for 10 min at 4°C, filtered through 0.2-μm syringe filter (Nalgene, Rochester, NY), and stored in aliquots at −80°C. The virus titer was determined by infecting CEFs with diluted virus stock. Both RCASBP(A)GFP and RCASBP(C)GFP virus stocks regularly reached a titer of 10^7 IU/ml. Stocks of RCASBP viruses were also generated by transfection of plasmid DNA by the calcium phosphate precipitation method (24). In standard transfections, 5 μg of purified plasmid DNA was introduced into DF-1 cells by the calcium phosphate precipitation method (33). Viral spread was monitored by assaying culture supernatants for ASLV capsid protein (CA) by enzyme-linked immunosorbent assay (ELISA) (49). Virus stocks were generated from cell supernatants cleared of cellular debris by centrifugation at $2,000 \times g$ for 10 min at 4°C and stored in aliquots at −80°C.

DNA constructs. The pTvc expression plasmid was constructed using PCR with the sequence encoding the Tvc receptor truncated after the glycine residue (Gly335) of the cytoplasmic B30.2 domain (see Fig. 2). The PCR product was generated using primers TVC6 and TVC3 (see below) and line H6 cDNA and was subsequently cloned into the EcoRI and BglII sites of the pSG5 eukaryotic 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* cDNA coding region.

A gene encoding a soluble form of the chicken Tvc receptor (*stvc-mIgG*) and a soluble form of the chicken Tvb^{S3} receptor (*stvb*^{S3}*-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 *stvc-mIgG* and *stvb*^{S3}*-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 sTvc-mIgG protein or the sTvbS3-mIgG protein were collected. The chicken sTva-mIgG protein was collected in supernatants from a stable DF-1 cell line expressing the *tvaSstva-mIgG* 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 *SU(A)-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 previ-

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

Cells	ASLV receptor(s)	ASLV titer (avg ± SD)[a]		
		RCASBP(C)AP	RCASBP(A)AP	RCASBP(B)AP
DF-1	Tva, Tvb, Tvc	$(2.4 \pm 0.6) \times 10^4$	$(4.5 \pm 3.3) \times 10^6$	$(4.2 \pm 1.2) \times 10^4$
NIL-Tvc	Tvc	$(7.9 \pm 1.5) \times 10^3$	<1	5.3 ± 2.5
NIL-Tva	Tva	<1	$(5.4 \pm 2.3) \times 10^4$	3.0 ± 2.6
NIL	None	<1	<1	1.0 ± 1.0

[a] 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.

ously (24). The receptor subgroup of the viral envelope glycoprotein is given in parentheses in these virus designations.

The *tvc* knockout constructs were generated by insertion of isogenic homology regions from the 5′ and 3′ ends of the DT40 *tvc* 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 SalI digestion, and ligated into unique KpnI and SalI 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 *tvc*. 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 pLOX*tvc*Bsr, the blasticidin S resistance gene was replaced with the puromycin resistance gene from pLoxPuro by using the SalI and XhoI sites to create pLOX*tvc*Puro (4).

ALV AP assay. For AP assays, cell 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 Dodgson (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 Pharmaceuticals) (5). BAC DNAs were amplified and purified with the QIAGEN large-construct kit (QIAGEN Inc., Valencia, Calif.). BAC CH261-100I9 DNA was digested with XhoI and SfuI, and a 15-kb DNA fragment containing the *tvc* locus was purified by agarose gel electrophoresis and subcloned into the SalI and NarI sites of plasmid pUC18 (55).

Reverse transcription-PCR (RT-PCR) and PCR. The following oligonucleotide primers were used in this study (all oligonucleotides are written 5′ to 3′): TVC1, CTGACCCTGTGGCCGTGGCTGTG; TVC2, TGGGGATCTCCTCA TCCACGCTG; TVC3, ctagagatcTGCCATCATCAGTCAGGACC; TVC4, CT CCTGGTCCCCATAGTGGTCCTG; TVC5, GGGTCGCTCTGGATTATGA AGTGG; TVC6, atccgaatTCCATGgAGA*c*GATGTTTTTTGGCTG; TVC7, CT CGCTGGCAGAGCCAGGAC; TVC8, AGGCTGGTTGGTTTAGCAGTAG; TVA1, CATGGTGCGGTTGTTGGAG; TVA2, GGGATCGCGCGGCTCCG AAC; TVB1, CAGACCTCCAGAAGCCAGAC; TVB2, CCCAGGCACTTGG GAAAG; GAPDH1, CCATGACAACTTTGGCATTG; GAPDH2, TCCCCAC AGCCTTAGCAG; KO1L, agtcgtcgacCAGGCAAAGAAGGGAATGAA; KO1R, agtcgtcgacACAGTTGGGCAAGCCTGTAA; KO2L, agtactagtTTCTCT GCAGGTGACTGTGG; KO2R, agtactagtCTGCTGGCTGCTGTAGTCTG; HR, ATACACTGGGGAGGTTCAGAAGC; BS1, CGATTGAAGAACTCAT TCCACTCAAATATACCC; PU4, CAGCGCCCGACCGAAAGGAGCGCAC GACC; and IGL, AGGCAGGTATAACGCCCTCT. In these sequences, the lowercase letters designate sequences at the 5′ ends of the primers that are not complementary to the templates, and the restriction enzyme sites used for cloning of the PCR products are underlined (EcoRI in TVC3, BglII in TVC6, SalI in KO1L and KO1R, and SpeI in KO2L and KO2R). The two lowercase letters in italic in primer TVC6 indicate mismatches with the *tvc* sequence in inbred line H6. The mismatches arose by designing this oligonucleotide based on the *tvc* sequences from expressed sequence tag data, which differ at these two positions from the *tvc* sequence in inbred line H6.

Total RNA was isolated from CEFs or from chicken tissues by using the TRI reagent (Sigma-Aldrich, St. Louis, Mo.) according to the manufacturer's instructions. RNA samples (1 μg) were converted to cDNA by using the Moloney murine leukemia virus reverse transcriptase (Promega, Madison, Wis.) with an oligo(dT) primer. PCR amplifications of regions of the *tvc* cDNA were performed using the Accu Taq polymerase (Sigma-Aldrich, St. Louis, Mo.) according to the manufacturer's instructions. The following PCR conditions were used: 2 min at 94°C; 33 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 initial amplification of a 1,257-bp fragment containing most of the *tvc* open reading frame used primers TVC1 and TVC2 and an annealing temperature (T_A) of 58°C. The 5′ and 3′ end regions of the *tvc* transcript were determined from rapid amplification of cDNA ends (RACE) reactions (see below). The entire coding region of *tvc* was amplified using primers TVC7 and TVC8 located in the 5′ and 3′ untranslated region sequences (T_A = 56°C). The truncated form of Tvc contained in the pTvc plasmid was generated using primers TVC6 and TVC3 (T_A = 56°C).

To compare the expression levels of *tva*, *tvb*, and *tvc* 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 bellow), and 40 s at 72°C; and a final extension of 3 min at 72°C. Using these PCR conditions, *tvc*, *tva*, *tvb*, and *gapdh* 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 *gapdh* transcripts in individual samples. The primer combinations and annealing temperatures used for this analysis were as follows: for amplification of *tvc* cDNAs, primers TVC6 and TVC4 and a T_A of 55°C; for amplification of *tvb* cDNAs, primers TVB1 and TVB2 and a T_A of 58.5°C; for amplification of *tva* cDNAs, primers TVA1 and TVA2 and a T_A of 57°C; and for amplification of *gapdh* cDNAs, primers GAPDH1 and GAPDH2 and a T_A of 58°C.

RACE. 5′- and 3′-RACE reactions were used to generate a complete *tvc* sequence. The SMART RACE (Clontech, Palo Alto, CA) procedure was employed according to the manufacturer's instructions. One microgram of total RNA from line H6 CEFs was converted to cDNA by using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, Wis.) and used as a template for both RACE reactions. The *tvc*-specific antisense primers TVC2, TVC4, and TVC6 were used for the 5′-RACE. The *tvc*-specific sense primers TVC1 and TVC5 were used for the 3′-RACE. Both 5′- and 3′-RACE reactions yielded single distinct PCR products whose nucleotide sequences were determined directly by sequencing with the TVC4 and TVC5 primers, respectively.

Assays for Tvc receptor function. Line L15 CEFs were seeded at 3×10^5 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 pTvc 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 pMC1 NEO poly(A) (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.

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 Pfam 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^{Cre1} cells (5×10^6 in 400 μl of medium) were electroporated (25 μF and 700 V applied in a 4-mm cuvette) with 20 μg of linearized pLOX*tvc*Bsr by using the Gene Pulser Xcell (Bio-Rad). After electroporation, 5×10^4 cells were plated per microtiter well in 100 μl of growth medium. Twenty-four hours later the medium was changed to growth medium supplemented with15 μg/ml blasticidin S (Invitrogen). 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 pLOX*tvc*Puro as described above and selected for puromycin resistance in medium supplemented with 1 μg/ml puromycin (Sigma). Puromycin-resistant clones were checked for resistance to blasticidin S and for homologous recombination of the pLOX*tvc*Puro construct. Homologous recombination of pLOX*tvc*Bsr was detected using primer HR, complementary to nucleotides 4181 to 4159 5′ from the *tvc* ATG, and primer BS1, complementary to sequence in the blasticidin S resistance gene (4). Homologous recombination of pLOX*tvc*Puro was detected using primer HR and primer PU4 within the puromycin resistance gene sequence. Nontargeted *tvc* genes were detected using primer HR and primer IGL 3′ (the end of 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 mIgG 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 fragment per ml.

Immunoprecipitations and Western immunoblot analysis. The sTvc-mIgG, sTva-mIgG, and sTvbS3-mIgG proteins were immunoprecipitated separately with anti-mouse IgG-agarose beads (Sigma) for ≥1 h at 4°C, and the SU(C)-rIgG, SU(A)-rIgG, and SU(B)-rIgG 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-mouse IgG (heavy plus light chains [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 Larco (Quality Biological, Inc.) and washed with Dulbecco'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-mIgG or SU-rIgG fusion proteins on ice for 30 min. The stable DF-1 cell lines TF/sTvaS-18 (expressing chicken sTva-mIgG), TF/sTvb^{S3}-8 (expressing sTvb^{S3}-mIgG), TF/SU(A)-19 [expressing SU(A)-rIgG], and TF/SU(B)-12 [expressing SU(B)-rIgG]; RCASBP(A)*stvc-mIgG*-infected DF-1 cells (expressing sTvc-mIgG); and RCASBP(A)*SU(C)-rIgG*-infected DF-1 cells [expressing SU(C)-rIgG] were the sources of the receptor-mIgG or SU-rIgG 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–Ig-phycoerythrin 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.

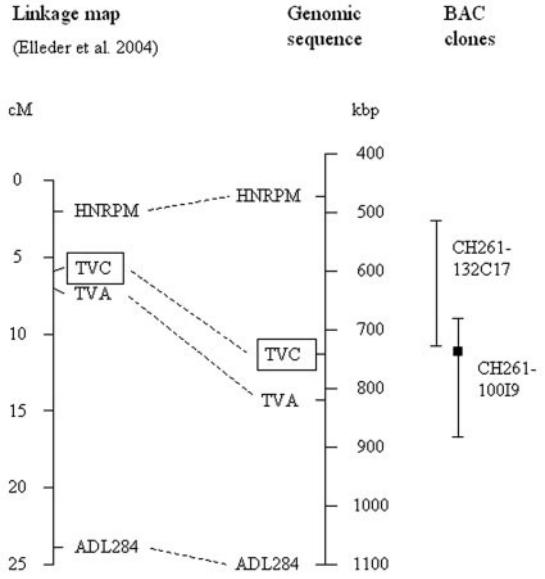

FIG. 1. Alignment of the genetic linkage map and the genome sequence of a region of chicken chromosome 28. A portion of chicken chromosome 28 is shown, depicting the positions of *tva*, *tvc*, and the two nearest genetic markers. Corresponding loci are connected by dotted lines. The positions of BACs CH261-100I9 and CH261-132C17, which tested positive and negative for the presence of *tvc*, respectively, are shown on the right. The small black square depicts the position of the 15-kbp genomic fragment that contained the *tvc* gene.

***K_D* 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, $f(y) = M/[1 + e^{-r(\log x - \log K_D)}]$, where y is the mean fluorescence, M is the maximum fluorescence, r is the rate, x is the concentration of the receptor-mIgG or SU-rIgG fusion protein, and K_D is the dissociation constant, which is defined as the concentration of the receptor-mIgG or SU-rIgG fusion protein at half-maximal binding (30).

Nucleotide sequence accession number. The complete nucleotide sequence of the *tvc* 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 *tvc* 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 CEFs, which are resistant to ASLV(C) infection, and challenged with RCASBP(C)GFP, an ASLV(C) virus containing 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 *tvc* 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 *tvc* 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 *tvc* 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 *tvc* 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 *tvc* cDNA (data not shown). The resulting sequences were assembled into a *tvc* cDNA consensus sequence (Fig. 2). This *tvc* cDNA consensus sequence was verified by PCR amplification of full-length *tvc* cDNAs by using primers based on the ends of the consensus sequence (data not shown). The *tvc* 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 *tvc* 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 *tvc* gene were determined by comparing the *tvc* cDNA nucleotide sequence to the draft chicken genome sequence (Fig. 2). All *tvc* 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 *tvc* cDNA confers susceptibility to ASLV(C) infection. To test the ability of the *tvc* cDNA to confer susceptibility to ASLV(C) infection, the expression plasmid pTvc was constructed from a fragment of the *tvc* 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 *tvc* cDNA at a time when a cDNA encoding the complete B30.2 cytoplasmic domain was not yet cloned. This *tvc* 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 *tvc* 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.

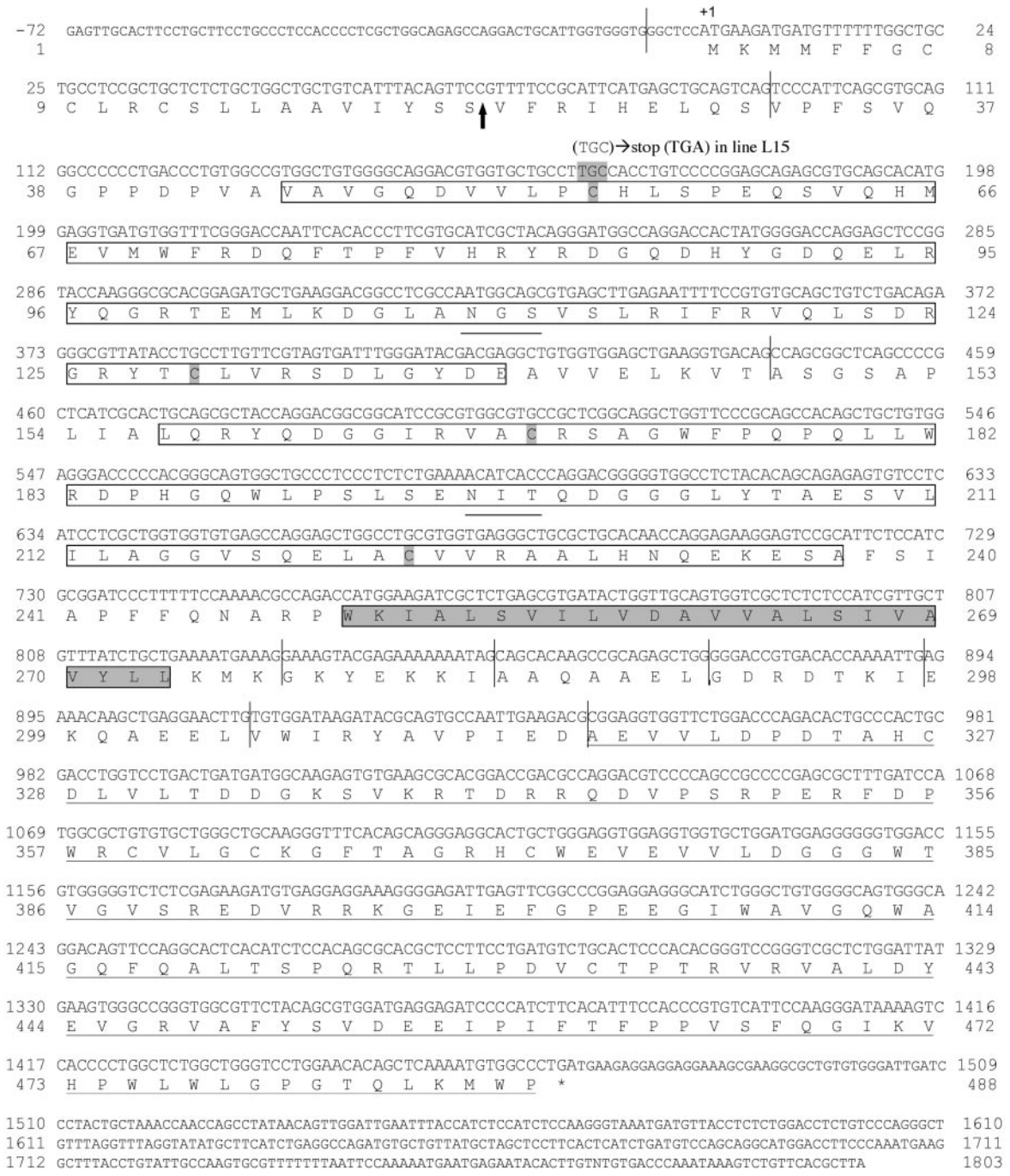

FIG. 2. The nucleotide sequence of the *tvc* cDNA. The nucleotide and deduced amino acid sequences of Tvc are shown, with the smaller nucleotide letters indicating the 5′ and 3′ untranslated regions of the *tvc* mRNA. The open boxes in the extracellular region of Tvc are the IgV and IgC domains, the shaded box is the transmembrane domain, and the long underlined region in the intracellular domain is the region of Tvc homologous to the B30.2 domain. The predicted leader peptidase cleavage site is marked by an arrow and the exon junctions by vertical lines. The cysteine residues in both Ig domains are shaded, and the site of the mutation in the line L15 *tvc* cDNA is highlighted. The two potential N-linked glycosylation sites are also underlined.

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

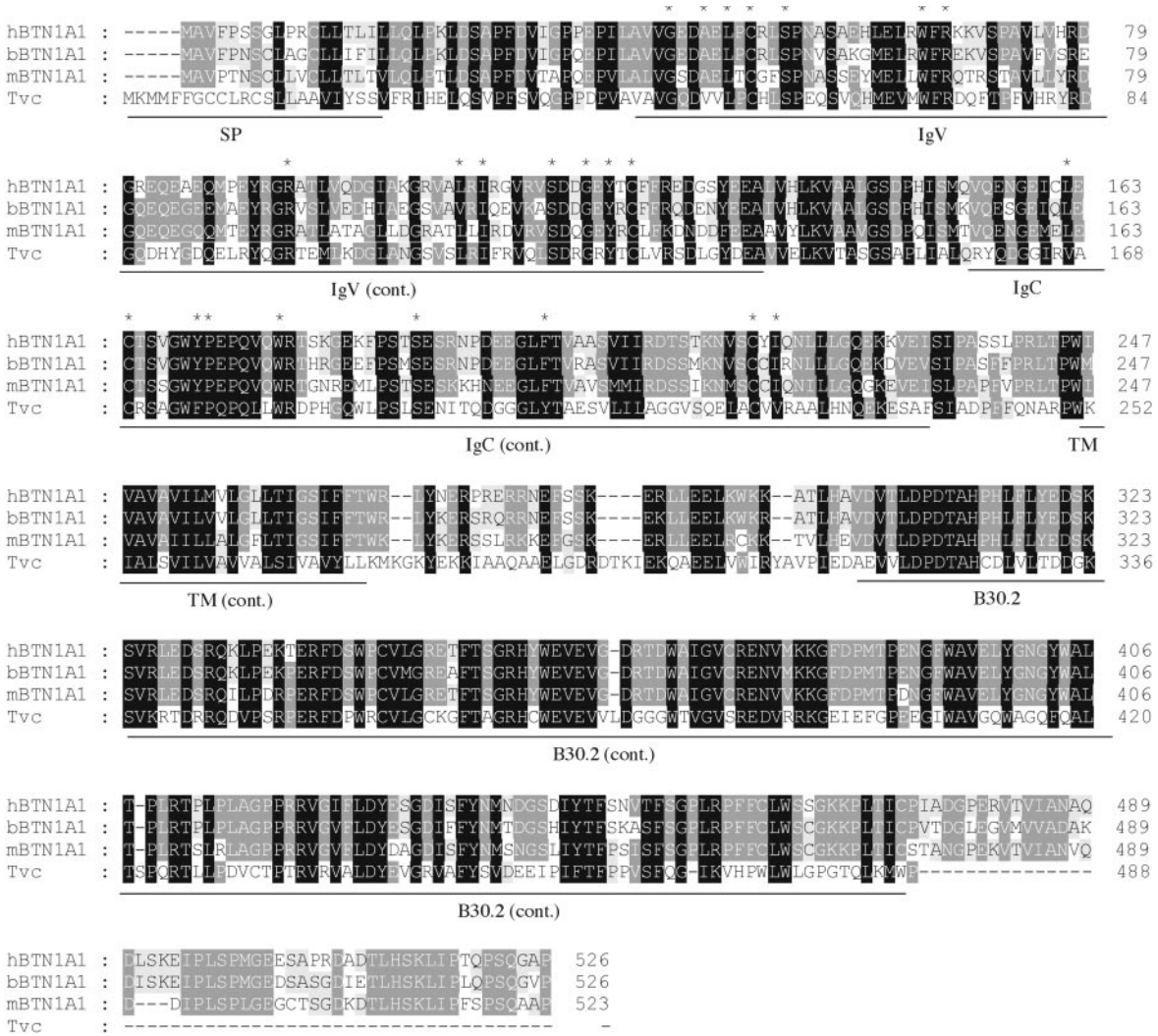

FIG. 3. Alignment of Tvc with human, bovine, and murine butyrophilins. The amino acid sequence of Tvc was aligned with those of the three proteins with the highest homology from the BLAST search, three butyrophilins classified as subfamily 1 member 1 (BTN1A1): human (hBTN1A1, accession number NP_001723.1), bovine (bBTN1A1, accession number NP_776933.1), and mouse (mBTN1A1, accession number AAH31459.1). From this analysis, homologous domains were predicted in Tvc (underlined); SP indicates the signal peptide and TM the transmembrane domain. Amino acid residues that are conserved within the IgV and IgC1 folds are indicated with asterisks (18). The protein alignment was performed using the ClustalW program and then highlighted in gray scales using the Genedoc program.

env). 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 $tvc^{-/-}$ 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$tvc^{-/-}$ 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$tvc^{-/-}$ cells were highly resistant to RCASBP (C)GFP (Fig. 5B, panel d) but still susceptible to infection by

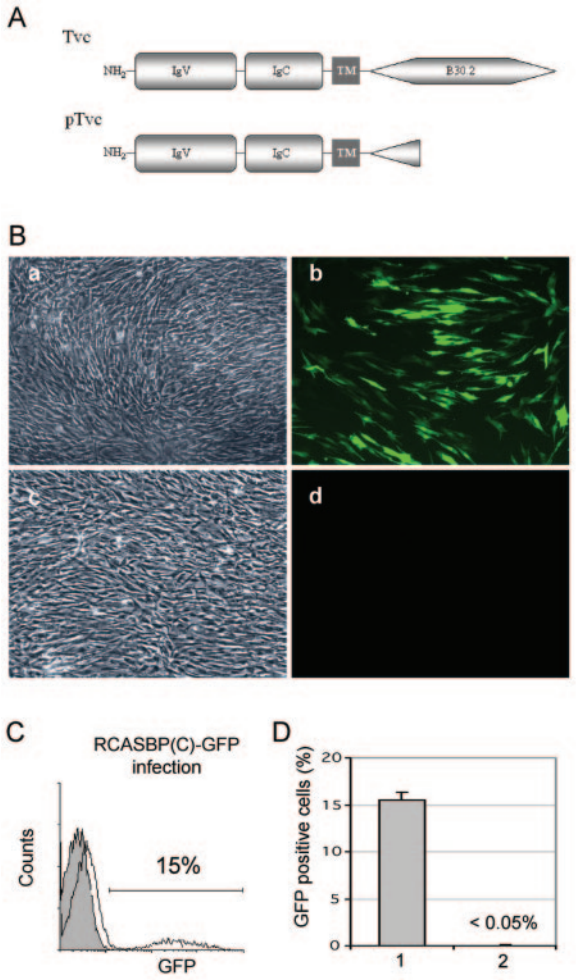

FIG. 4. Expression of the *tvc* cDNA allows ASLV(C)s to infect resistant chicken cells. Line L15 CEFs were transfected with the plasmid pTvc and subsequently infected with RCASBP(C)GFP virus. As a negative control, the pTva plasmid was transfected into line L15 CEFs and challenged with RCASBP(C)GFP virus. The infected GFP-positive cells were quantified by flow cytometry. (A) Schematic diagrams of the full-length Tvc protein and of the truncated product expressed from the pTvc plasmid. (B) Images from phase contrast microscopy (a and c) and fluorescence microscopy (b and d) of L15 CEFs transfected with pTvc (a and b) or the control pTva plasmid (c and d). (C) A representative histogram depicting flow cytometry analysis of cells transfected with pTvc (open curve) or a control plasmid (shaded curve). The relative fluorescence is plotted against the cell count; the percentage of cells in which the fluorescence activity was above that measured in the control is indicated. (D) Percentage of GFP-positive cells from FACS analysis; the averages and standard deviations from two experiments performed in triplicate are shown. L15 CEFs were transfected with pTvc (bar 1) or control plasmid (bar 2).

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-

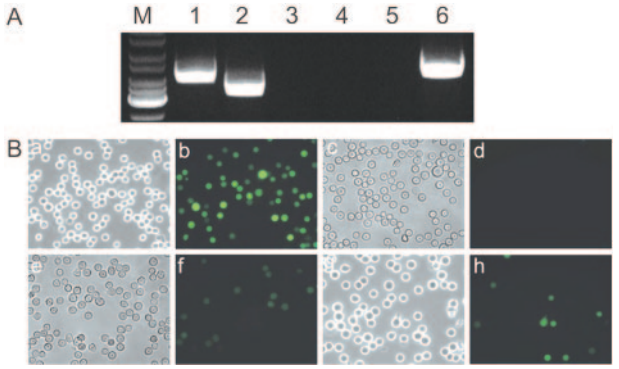

FIG. 5. Targeted deletion of *tvc* renders DT40 cells resistant to ASLV(C) but not to ASLV(B) infection. (A) PCR detection of homologous recombination of knockout constructs and *tvc* in DT40*tvc*$^{-/-}$ (lanes 1 to 3) and parental (lanes 4 to 6) DT40 cells. Homologous recombination of the pLOX*tvc*Bsr construct was detected using primers HR and BS1 (lanes 1 and 4), homologous recombination of the pLOX*tvc*Puro knockout construct was detected using primers HR and PU4 (lanes 2 and 5), and intact *tvc* was detected using primers HR and IGL (lanes 3 and 6). M, DNA marker ladder. (B) Phase-contrast (a, c, e, and g) and fluorescence (b, d, f, and h) microscopy of infected cells. Parental DT40 cells (a, b, e, and f) and DT40*tvc*$^{-/-}$ cells (c, d, g, and h) were infected by RCASBP(C)GFP (a to d) or RCASBP(B)GFP (e to h) virus.

mIgG, and sTvb^{S3}-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 sTvb^{S3}-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)-rIgG and SU(A)-rIgG, but not SU(B)-rIgG, to uninfected DF-1 cells (Fig. 6E). The binding affinities measured for the SU(C)-Tvc interaction (3.93 nM) and the SU(A)-Tva interaction (0.84 nM) by using this approach were both ~10-fold lower than those we obtained using the soluble receptor approach (Table 2).

The *tvc*^{r} 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

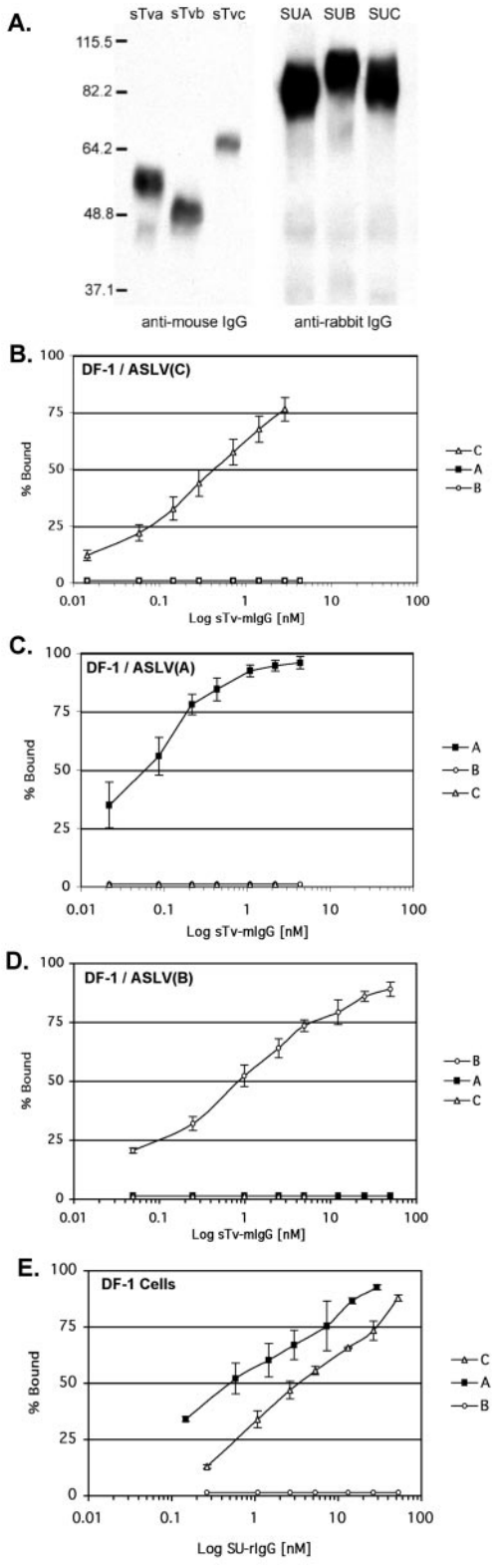

FIG. 6. Binding affinity of the ASLV envelope glycoproteins for ASLV receptors. (Panel A) Western immunoblot analysis of the soluble forms of the Tvc receptor sTvc-mIgG (sTvc) the chicken Tva receptor TvaSsTva-mIgG (sTva), and the Tvb receptor $sTvb^{S3}$-mIgG (sTvb) immunoprecipitated with anti-mouse 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.

(TGA) (Fig. 2). This would produce a severely truncated Tvc receptor, the signal peptide plus 32 amino acid residues of the mature protein, and abolishes its use as an ASLV(C) receptor. We are designating the chicken line L15 *tvc* gene tvc^r. 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., tva^r and tvb^{S3}) or eliminate the expression of the receptor (e.g., tvc^r, tva^{r2}, and tvb^r). 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-

TABLE 2. Estimated binding affinities of soluble forms of the ASLV receptors for ASLV envelope glycoproteins expressed on the surface of infected DF-1 cells and of soluble forms of the ASLV surface glycoproteins for endogenous levels of the ASLV receptors expressed on DF-1 cells

Cells	Apparent K_D (nM)[a]					
	Receptor			Surface glycoprotein		
	sTvc-mIgG	sTva-mIgG	sTvb^{S3}-mIgG	SU(C)-rIgG	SU(A)-rIgG	SU(B)-rIgG
DF-1	—[c]	—	—	3.93 ± 0.26	0.84 ± 0.28	NDB[b]
DF-1/ASLV(C)	0.55 ± 0.20	NDB	NDB	—	—	—
DF-1/ASLV(A)	NDB	0.05 ± 0.02	NDB	—	—	—
DF-1/ASLV(B)	NDB	NDB	0.90 ± 0.25	—	—	—

[a] Apparent K_D 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.

group C ASLVs, is related to a member of the immunoglobulin protein Ig family; Tva, the receptor for subgroup A ASLVs, is related to the LDLR family; and Tvb, the receptor for the subgroup B, D, and E ASLVs, is related to the TNFR family. Except for the fact that all three proteins are ASLV receptors, the proteins have no obvious sequence or structural homology, nor do they appear to be functionally related. However, the ASLV SU glycoproteins have evolved the ability to bind each of the proteins and carry out the multistep process leading to fusion of the viral and cellular membranes. Although it has not yet been proven experimentally, all of the subgroup A to E ASLVs likely require both receptor binding and low pH to complete the fusion process. This dual requirement is, as far as is now known, found only in the ASLV *env* proteins (6). It is likely that the requirement for low pH will require ASLV virions to enter through a particular cellular compartment, which could limit the choice of cellular proteins that could be acceptable ASLV receptors.

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. Third, each ASLV receptor protein 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.

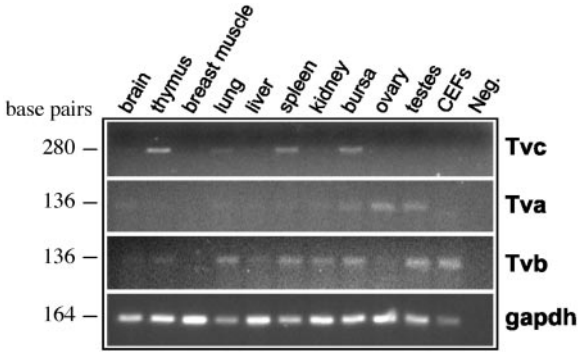

FIG. 7. RT-PCR amplification of *tva*, *tvb*, and *tvc* transcripts from various chicken tissues. The cDNA samples were prepared from various tissues of outbred Brown Leghorn chickens or from CEFs from inbred line M. PCR primers were designed to amplify short regions in the N-terminal regions of the *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.

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-rIgG 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-

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 SUs 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.

ACKNOWLEDGMENTS

This work was supported in part by grant 523/04/0489 from the Grant Agency of the Czech Republic, project AV0Z50520514 awarded by the Academy of Sciences of the Czech Republic, and National Institutes of Health grant AI48682 and the Mayo Foundation (to M.J.F.).

We thank Stephen Hughes and Jerry Dodgson for discussions and reading of the manuscript, Richard Crooijmans and Martien Groenen for an initial PCR screen of the Wageningen BAC library, Jerry Dodgson and Michael Romanov for providing BAC library filter sets and BAC clone DNAs, Jean-Marie Buerstedde and Hiroshi Arakawa for training in the DT40 knockout technology, and Lisa Stubbs for sharing unpublished chicken genomic sequence data. We thank all the personnel of the Koleč animal farm and Audelia Munguia for excellent technical assistance.

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