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Resistance to Infection by Subgroups B, D, and E Avian Sarcoma and Leukosis Viruses Is Explained by a Premature Stop Codon within a Resistance Allele of the *tvb* Receptor Gene

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Here we present the first molecular characterization of the defect associated with an avian sarcoma and leukosis virus (ASLV) receptor resistance allele, *tvb*^{r}. We show that resistance to infection by subgroups B, D, and E ASLV is explained by the presence of a single base pair mutation that distinguishes this allele from *tvb*^{s1}, an allele which encodes a receptor for all three viral subgroups. This mutation generates an in-frame stop codon that is predicted to lead to the production of a severely truncated protein.

Functionally distinct alleles of the autosomal *tva*, *tvb*, and *tvc* loci that encode avian sarcoma and leukosis virus (ASLV) receptors have been defined for chickens (reviewed in references 12 and 22). Two classes of *tva* and *tvc* alleles are associated with either susceptibility (*tva*^{s} and *tvc*^{s}) or resistance (*tva*^{r} and *tvc*^{r}) to infection by subgroup A ASLV or subgroup C ASLV, respectively. The *tvb* locus is more complex since two chicken alleles of *tvb* that encode distinct ASLV receptors have been defined: *tvb*^{s1} for viral subgroups B, D, and E and *tvb*^{s3} for subgroups B and D (1, 2, 9, 20). In addition, there is another type of allele (*tvb*^{r}) that cannot support entry by any of these ASLV subgroups (12). For each ASLV receptor gene, alleles that confer susceptibility to viral infection are dominant over those associated with resistance. Therefore, the only lines of chickens that are resistant to infection by subgroups A through D ASLVs are those that are homozygous for the resistance alleles of the cognate receptor gene. Resistance to subgroup E viral infection is more complicated because of the existence of endogenous ASLV elements in the chicken germ line which encode subgroup E ASLV-specific envelope (Env) proteins that interfere with the function of the TVB^{S1} receptor (1, 7). The recessive nature of the *tva*^{r}, *tvb*^{r}, and *tvc*^{r} alleles rules out the possibility that their products interfere, in a dominant-negative manner, with those encoded by the corresponding susceptibility alleles. Instead, the defect(s) associated with these resistance alleles may be due either to their lack of expression or to the existence of specific amino acid substitutions in the corresponding proteins that abolish viral receptor function.

The *tva*^{s} allele encodes a low-density lipoprotein (LDL) receptor-related protein that is a cellular receptor for ASLV subgroup A (5, 6, 26). The TVA protein contains an approximately 40-amino-acid long LDL-A module that harbors the major viral interaction determinants (16, 17, 27, 28). The molecular defect that is associated with the *tva*^{r} allele has not yet been defined, but a preliminary characterization of this allele has documented no obviously debilitating defects within the LDL-A domain (5).

TVB is a tumor necrosis factor receptor (TNFR)-related death receptor that is most similar to the mammalian TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 (1). TVB contains three extracellular cysteine-rich domains and a cytoplasmic death domain which can activate apoptosis (8, 9). Several ASLV subgroups that utilize TVB exhibit an acute cytopathic effect. The death-promoting activity of TVB may contribute to the cell death that is observed in cultures that have been infected by subgroups B and D of ASLV (8, 9, 23, 24).

In order to understand the molecular basis for the resistance to viral infection that is associated with the *tvb*^{r} allele, we have characterized the nature of the defect. This analysis has revealed that the *tvb*^{r} mRNA harbors a premature stop codon that is predicted to lead to the production of a severely truncated protein.

The *tvb*^{r} allele is expressed as a normal-sized mRNA transcript. Samples of mRNA were prepared from chicken embryo fibroblasts (CEFs) with distinct *tvb* genotypes: line 0 (*tvb*^{s3}/*tvb*^{s3}), line 15_B1 (*tvb*^{s1}/*tvb*^{s1}), and line 7_2 (*tvb*^{r}/*tvb*^{r}) (provided by the Avian Disease and Oncology Laboratory, East Lansing, Mich.) (4). Approximately 1 μg of each mRNA sample, along with 5 μg of the 0.24- to 9.5-kb RNA ladder (Gibco/BRL), was subjected to electrophoresis in a 1.2% agarose gel containing 1.12% formaldehyde and morpholinepropanesulfonic acid (MOPS) buffer. The samples were then transferred to a nylon membrane (Amersham), and *tvb* mRNA was detected by probing with a ^{32}P-labeled 2.5-kb *Hin*dIII-*Hin*dIII DNA fragment derived from the *tvb* genomic clone pBK-9 as described previously (9).

This analysis revealed that the *tvb*^{r} mRNA transcript is similar in size to those of the functional *tvb*^{s3} and *tvb*^{s1} alleles (Fig. 1). Therefore, *tvb*^{r} does not display any obvious defects in gene expression, mRNA stability, or mRNA splicing.

The *tvb*^{r} allele encodes a prematurely terminated protein

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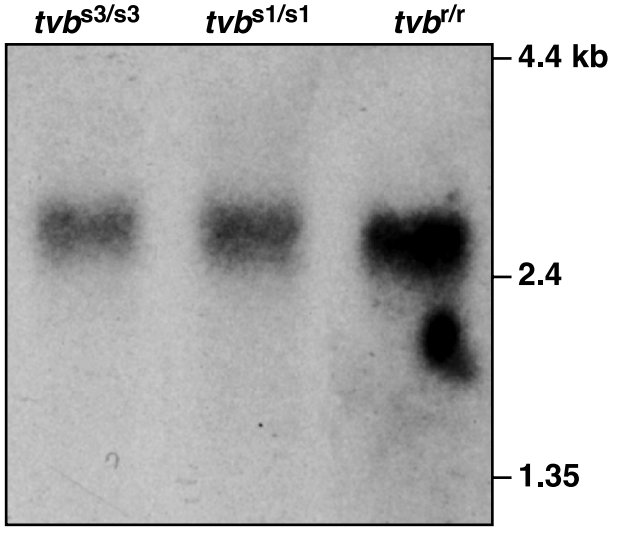

FIG. 1. The *tvb*^{r} allele is expressed as a normal-sized mRNA transcript. Northern blot analysis was performed with mRNA samples taken from CEFs with the indicated *tvb* genotypes. The nylon membrane was probed with a ^{32}P-labeled *tvb*-specific probe derived from the BK-9 *tvb* genomic DNA clone as described previously (9). Autoradiography was performed at −80°C with intensifying screens.

product. A reverse transcription-PCR (RT-PCR) amplification was used to further characterize the *tvb*^{r} mRNA from line 7_2 (*tvb*^{r}/*tvb*^{r}) CEFs. Two sets of oligonucleotide primers were used in this procedure, generating two overlapping cDNA fragments that spanned the entire length of the mRNA transcript (Fig. 2). Fragment 1 was generated by subjecting 500 ng of total RNA to an RT-PCR amplification protocol with the ProStar Ultra HF RT-PCR kit (Stratagene). RT was performed with an oligo(dT) primer, and the subsequent PCR amplification was performed with the sense primer oSK2 (5′-GCTAGCTAGCCGATGCGCTCAGCTGCGCTCCGG-3′) and the antisense primer oSK73 (5′-GGGGTACCCCGCT GGTATTTGGCACAGGGG-3′). The oSK2 primer corresponds to nucleotides 215 to 235 and the oSK73 primer corresponds to nucleotides 2190 to 2209 of the *tvb* cDNA (GenBank accession number AR071759) (Fig. 2). To facilitate subsequent cloning of the PCR-amplified products, *Nhe*I and *Kpn*I sites (underlined in the sequences) were incorporated at the 5′ ends of the oSK2 and oSK73 primers, respectively. A 1/10 fraction of the RT products was subjected to PCR amplification with *Pfu* Turbo DNA polymerase (Stratagene) for 40 cycles (94°C for 1 min, 65°C for 1 min, and 72°C for 3 min). The approximately 2-kb cDNA fragment 1, predicted to contain all of the putative *tvb*^{r} coding sequence, was subcloned into the pCI mammalian expression vector (Promega).

The SMART RACE cDNA amplification kit (Clontech) was used to generate cDNA fragment 2. The 5′ rapid amplification of cDNA ends-ready cDNA was synthesized from 500 ng of line 7_2 (*tvb*^{r}/*tvb*^{r}) RNA, and PCR was performed utilizing the Clontech SMART universal primer and the gene-specific antisense primer oSK20 (5′-GAAGGAAGGAAGCGGCCGCG CACTGCGTGTTCCTGGTGGGG-3′; the *Not*I site is underlined) corresponding to nucleotides 496 to 519 of the *tvb* cDNA (Fig. 2). cDNA fragment 2 was T/A cloned into the pT-Adv vector (Clontech). The overlapping cDNA fragments 1 and 2 spanned the entire length of the *tvb*^{r} mRNA, allowing us to analyze the full cDNA for any mutations (Fig. 2).

DNA sequence analysis of three independent clones of cDNA fragment 1 and nine independent clones of cDNA fragment 2 revealed only a single nucleotide difference between the open reading frames (ORFs) of *tvb*^{s1} and *tvb*^{r}. A cytosine residue located 172 nucleotides downstream of the start methionine codon in *tvb*^{s1} is replaced by a thymidine residue in *tvb*^{r} (Fig. 3). This change generates an in-frame stop codon (CAG→UAG). The presence of this mutation indicates that the *tvb*^{r} allele encodes a severely truncated protein product.

The point mutation in *tvb*^{r} generates a *Bfa*I restriction enzyme site that can be used diagnostically. The nucleotide difference between the ORFs of *tvb*^{r} and *tvb*^{s1} generated a *Bfa*I restriction enzyme site that is specific to the resistance allele (Fig. 3). To test whether the presence of this site could be used as a diagnostic marker for the presence of this allele, Southern blot analysis was performed with *Bfa*I-digested genomic DNA samples that were prepared from CEFs with different *tvb* genotypes. Using a panel of more than 50 independent restriction enzymes (not including *Bfa*I), Smith et al. had previously failed to identify any restriction fragment length polymorphism that could be used to distinguish between the resistance and susceptibility alleles of *tvb* (20).

For the present studies, 25-μg samples of genomic DNA from line 7_2 (*tvb*^{r}/*tvb*^{r}), line 0 (*tvb*^{s3}/*tvb*^{s3}), and line15_B1 (*tvb*^{s1}/*tvb*^{s1}) CEFs were digested overnight with 15 U of *Bfa*I (New England Biolabs, Inc.). Approximately 10 μg of each sample was then subjected to electrophoresis on a 1% agarose gel, and the samples were then transferred to a nylon membrane (Amersham). These samples were hybridized with a ^{32}P-labeled *tvb*-specific probe that was derived by PCR amplification with the *tvb*^{s3} cDNA clone pBK7.6-2 as the template DNA (9). The primers used for PCR amplification were the sense primer oSK2 (described above) and the antisense primer oSK65 (5′-GGCCAGCTGGTATTTGGCACAGGGG-3′), corresponding to nucleotides 2190 to 2209 of the *tvb* cDNA (GenBank accession number AR071759). The nylon membrane was incubated with the radiolabeled probe, washed at 65°C under standard conditions (10), and exposed to Kodak XAR-5 X-ray film

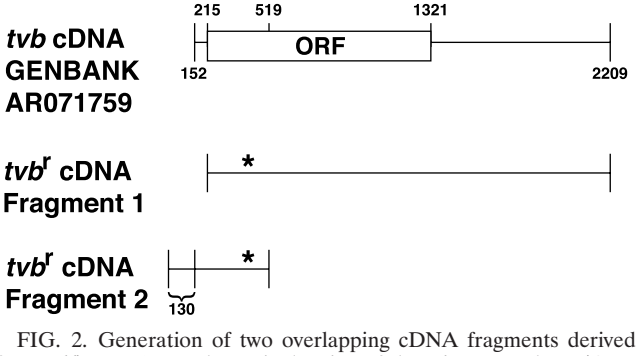

FIG. 2. Generation of two overlapping cDNA fragments derived from *tvb*^{r} mRNA. A schematic drawing of the *tvb* cDNA clone (GenBank accession number AR071759) is shown along with the location of two overlapping *tvb*^{r} cDNA fragments generated during this study. *tvb*^{r} cDNA fragment 2 contains a unique 5′ sequence derived by rapid amplification of cDNA ends which extends 130 bp beyond that reported previously. The point mutation is in the region of overlap and is indicated by an asterisk.

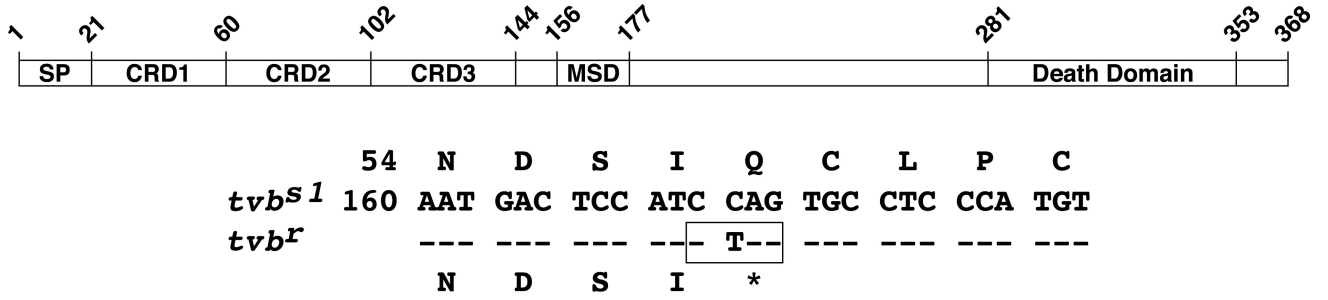

FIG. 3. The *tvb*^{r} allele contains a premature stop codon. A schematic drawing of TVB is shown depicting the signal peptide (SP), cysteine-rich domains (CRDs), membrane spanning domain (MSD), and cytoplasmic death domain. The nucleotide and amino acid sequences are also shown surrounding the single nucleotide change that distinguishes *tvb*^{r} from *tvb*^{s1} (numbered from the start methionine codon). The premature stop codon in *tvb*^{r} is indicated by an asterisk and a *Bfa*I site unique to *tvb*^{r} is shown in a box.

with intensifying screens at −80°C. This analysis revealed the presence of a 2.1-kb DNA fragment that is diagnostic for the *tvb*^{r} allele (Fig. 4). This DNA fragment serves as a useful marker for the *tvb*^{r} allele and supports the presence of the thymidine mutation in *tvb*^{r} DNA.

In this report we demonstrate the molecular defect associated with a resistance allele of an ASLV receptor gene. Resistance is due to the existence of a premature stop codon. This feature of *tvb*^{r} presumably either completely abolishes protein expression or leads instead to the generation of a severely truncated protein product that consists of only the 57 N-terminal amino acids (including the N-terminal signal peptide). Although mechanisms exist for translating in-frame stop codons (i.e., nonsense suppression), this is unlikely to apply in the case of *tvb*^{r} since the production of the full-length protein we predict would have ASLV receptor activity. The fact that *tvb*^{r} encodes an aberrant protein product most probably explains why this allele is recessive in nature. Indeed, we have not observed a TVB^R protein product of any size when the *tvb*^{r} cDNA fragment 1 cloned into the plasmid vector pCI (Promega) was transfected into 293 cells (data not shown). We conclude that the premature stop codon results in a lack of TVB expression and is a null allele of TVB.

Resistance to human immunodeficiency virus (HIV) infection has also been ascribed to a mutation at the level of the receptor (in this case a coreceptor) that leads to an aberrant protein product. Several naturally occurring mutations in the human CCR5 coreceptor gene have been shown to confer resistance to R5-tropic strains of HIV, including the CCR5delta32 allele, which harbors a 32-bp deletion leading to the production of a severely truncated protein product that does not support viral entry (13, 18). Individuals who lack CCR5 are less susceptible to infection by HIV, indicating an important role for this receptor at the bottleneck of transmission. Amino acid substitutions and/or posttranslational modifications that abolish the function of other retroviral receptors have also been described as barriers to virus transmission both among and between species (11, 15, 21, 25).

The ORFs of *tvb*^{s1} and *tvb*^{s3} were previously characterized, and they were found to differ only at nucleotide residue 184 (a thymidine or an adenosine, respectively). As a consequence, residue Cys-62 of TVB^{S1} is replaced by a serine in TVB^{S3} (Fig. 3). The corresponding residue of *tvb*^{r} is a thymidine, leading us to propose that this resistance allele evolved from *tvb*^{s1} during selective breeding among the commercial chicken population to confer resistance to subgroup B ASLV-induced lymphoid leukosis (4). As a consequence, the frequency of this allele and its homozygosity in the commercial chicken population is widespread.

The simple interpretation of the fact that chickens homozygous for *tvb*^{r} are viable is that this receptor (and by inference its ligand) is not essential for the life of these birds. However, TVB is most closely related to the mammalian TRAIL receptors DR4 and DR5. TRAIL activity has been linked to lymphocyte death and activation, antiviral immune defenses, and tumor surveillance (reviewed in reference 14). TRAIL has a complex relationship with its receptors, which is underscored by the presence of multiple signaling-competent and decoy receptors in humans and mice (3). Although the loss or dysregulation of TNFR-related proteins and/or their cognate ligands is typically associated with severe pathological consequences, it may be that the loss of TVB can be compensated for by the expression of another TNFR-related receptor that can bind to the putative TVB ligand. Two naturally occurring mutations in human Fas involving prematurely terminated Fas polypeptides (one with only the first 57 and another with only

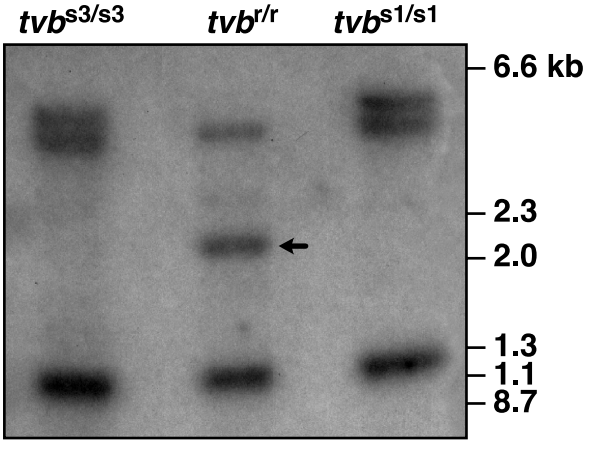

FIG. 4. A 2.1-kb *Bfa*I restriction fragment is diagnostic of the *tvb*^{r} allele. *Bfa*I-digested genomic DNA samples prepared from CEFs with the *tvb* genotypes indicated were subjected to Southern blot analysis with a ^{32}P-labeled *tvb*-specific DNA probe under standard conditions. Autoradiography was performed for 10 days at −80°C with intensifying screens. The position of the 2.1-kb DNA fragment that is unique to *tvb*^{r} is indicated with an arrow.

the first 62 amino acids of the mature Fas protein translated) contain a ligand-independent assembly domain and have been shown to dominantly interfere with normal Fas function (19). Because the effect of *tvb*^{r} on virus entry is recessive, we conclude that this truncated TVB^R polypeptide, if it is expressed, cannot dominantly interfere with virus entry via full-length TVB receptors. However, it is not clear whether the short putative TVB^R polypeptide would exert any negative effect on the natural function of the receptor, a possibility that can only be addressed when the TVB ligand has been identified and the normal physiological function of the receptor is known.

Nucleotide sequence accession number. The sequences for cDNA fragment 1 and cDNA fragment 2, which were generated during this study, have been submitted to GenBank and assigned accession numbers AF507016 and AF507017, respectively.

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