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Genetic Analysis of the Rous Sarcoma Virus Subgroup D *env* Gene: Mammal Tropism Correlates with Temperature Sensitivity of gp85

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Subgroup D avian sarcoma and leukosis viruses can penetrate a variety of mammalian cells in addition to cells from their natural host, chickens. Sequences derived from the gp85-coding domain within the *env* gene of a mammal-tropic subgroup D virus (Schmidt-Ruppin D strain of Rous sarcoma virus [SR-D RSV]) and a non-mammal-tropic subgroup B virus (Rous-associated virus type 2) were recombined to map genetic determinants that allow penetration of mammalian cells. The following conclusions were based on host range analysis of the recombinant viruses. (i) The determinants of gp85 that result in the mammal tropism phenotype of SR-D RSV are encoded within the 160 codons that lie 3′ of codon 121 from the corresponding amino terminus of the gp85 protein. (ii) Small linear domains of the SR-D RSV gp85-coding domain placed in the subgroup B background did not yield viruses with titers equal to that of the subgroup D virus in a human cell line. (iii) Recombinant viruses that contained subgroup D sequences within the *hr*1 variable domain of gp85 showed modest-to-significant increases in infectivity on human cells relative to chicken cells. A recombinant virus that contained three fortuitous amino acid substitutions in the gp85-coding domain was found to penetrate the human cell line and give a titer similar to that of the subgroup D virus. In addition, we found that the subgroup D virus, the mutant virus, and recombinant viruses with an increased mammal tropism phenotype were unstable at 42°C. These results suggest that the mammal tropism of the SR-D strain is not related to altered receptor specificity but rather to an unstable and fusogenic viral glycoprotein. A temperature sensitivity phenotype for infectivity of mammalian cells was also observed for another mammal-tropic avian retrovirus, the Bratislava 77 strain of RSV, a subgroup C virus, but was not seen for any other avian retrovirus tested, strengthening the correlation between mammal tropism and temperature sensitivity.

Envelope viruses, including retroviruses, enter host cells by either receptor-mediated endocytosis or direct fusion at the cell surface (reviewed in reference 43). Although the mechanism of viral penetration is reasonably well understood for influenza virus (44), the mechanism of penetration for most other enveloped viruses is less clear. Members of the avian sarcoma and leukosis virus (ASLV) group of retroviruses fuse in a pH-independent manner, suggesting entry at the plasma membrane (15). ASLV isolates use receptors on chicken cells to mediate fusion. Certain ASLV isolates can also infect cells from other galliform species and even mammalian cells. Therefore, the ASLV group of viruses provides a unique genetic system to analyze cell receptor-viral glycoprotein interaction and cell penetration by a group of highly related viruses.

ASLV isolates obtained from chickens have been classified into five subgroups, A through E, based on three distinguished phenotypes: the host range of the virus, viral glycoprotein interference of infection (41), and cross-neutralization patterns with viruses of the same subgroup (39, 40). Three dominant loci, *tv-a*, *tv-b*, and *tv-c*, have been identified in chickens as determinants of susceptibility to subgroup A, B, and C ASLV, respectively (9, 10, 29). These loci probably encode the receptors used by the viruses to enter cells. The *tv-b* locus also mediates susceptibility to subgroup D and E ASLV in chicken cells (reviewed in reference 19). The viral sequences that encode important determinants of subgroup specificity reside in a region of the viral genome encoding the *env* gene product gp85 (5, 6, 12, 13, 18). Nucleotide sequence (and predicted amino acid sequence) comparisons of gp85s from different ASLV subgroup viruses have revealed specific regions of divergence along the primary amino acid sequence of gp85 (5, 6, 12, 13). These variable regions (termed *vr*1, *vr*2, *hr*1, *hr*2, and *vr*3) reside essentially within the same relative positions of the gp85-coding domain, regardless of the subgroups compared. The variable regions are believed to contain the major determinants that define subgroup specificity in ASLV.

Subgroup D viruses can infect a variety of mammalian cells in addition to susceptible chicken cells (reviewed in reference 42). We have previously shown that genetic determinants which permit a subgroup D isolate of ASLV (Schmidt-Ruppin strain of Rous sarcoma virus subgroup D [SR-D RSV]) to penetrate a variety of mammalian cells reside in the gp85-coding domain of ASLV (6). To map more precisely the mammal tropism determinants of SR-D RSV, we constructed a series of recombinant viruses between the SR-D RSV gp85-coding domain and a subgroup B virus (Rous-associated virus type 2 [RAV-2]) that does not penetrate mammalian cells. By placing small segments of the subgroup D gp85-coding sequence into the corresponding positions of a subgroup B virus, we were unable to generate a virus that gave an equivalent titer in a mammalian cell line compared with the titer of the subgroup D virus. However, changes in the *hr*1 variable domain correlated with an increased mammal tropism phenotype, including a mutant which had a mammal tropism phenotype similar to that of the subgroup D parent. The extent of mammal tropism correlated with temperature sensitivity in all of the viruses

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that displayed a mammal tropism phenotype. This correlation was extended to include another naturally occurring mammal-tropic variant, Bratislava 77 strain (B77) subgroup C RSV (4, 14). We propose that glycoprotein instability is an important determinant in the extended host range of mammal-tropic avian retroviruses.

MATERIALS AND METHODS

Cells and viruses. C/O chicken embryos (susceptible to all ASLV subgroup viruses) were obtained from the Regional Poultry Research Laboratory (East Lansing, Mich.) and stored frozen as primary or secondary fibroblast cells. Preparation and maintenance of chicken embryo fibroblast (CEF) cells have been described previously (5). QT6, a chemically transformed quail cell line (26), cells were maintained as described previously (5). U-251 MG cells (3) were obtained from M. Cloyd and maintained in Dulbecco minimum essential medium supplemented with 10% fetal bovine serum. Viral genomes were obtained as molecular clones. λ-RAV-2, a DNA clone containing the RAV-2 genome, was obtained from G. Payne and H. E. Varmus (28). The SR-D RSV genome was obtained from D. Shalloway (32) as DNA plasmid clone pSRD-11. pANV-A (27) contains the structural genes of SR-A RSV (11), as well as the Neo^r-encoding gene, the simian virus 40 origin of replication, and the origin of replication of pSV2neo (33), in place of the v-*src* gene of SR-A RSV. The parental plasmid of pANV-A was replication-competant vector p779NC327AC28F (17). The *Kpn*I-to-*Sal*I *env* fragment from pSRD-11 was cloned into the equivalent position in pANV-A to create pANV-D, which was obtained from W. Osheroff. A similar strategy was used to generate pANV-B8, pANV-C, pANV-A1, and pANV-E (5, 6). Transfection of each pANV into CEF cells gave rise to the corresponding avian Neo^r virus (ANV).

B77 RSV was rescued from the B31 rat cell line (38) by fusion with CEF cells in the presence of polyethylene glycol (34). Virus released after cell fusion was propagated in CEF cells and then used to rescue a Neo^r gene-containing replication-defective viral genome present in a clonal derivative of QT6 cells (27a).

Site-directed mutagenesis; plasmids and bacteria. The 1.1-kb *Kpn*I-to-*Sal*I *env* fragment of λ-RAV-2 was cloned into M13mp19 to yield M13RAV-2. The bacteriophage was propagated in *Escherichia coli* JM101 (25). Site-directed mutagenesis (22) was performed with the M13RAV-2 phage template that was passed through *E. coli* CJ236 *dut ung* (20, 22) to create a uracil-containing DNA template. Either chemically synthesized oligonucleotides or denatured restriction enzyme fragments served as primers in the mutagenesis procedure. Two restriction enzyme fragments containing *env* sequences, an *Xho*I-to-*Sal*I (666-bp) fragment and a *Pst*I-to-*Sal*I (510-bp) fragment, were digested with *Rsa*I or *Sau*3A (Life Sciences, Inc., Gaithersburg, Md.) to give longer subgroup D-specific primers. The mutagenesis protocol using oligonucleotide primers was performed essentially as previously described (22). *E. coli* JM101 or DH5αF′ was transformed with the extended-ligated reaction products, and recombinant *env* clones were identified by DNA sequence analysis.

The double-stranded replicative form of recombinant M13 clones carrying the mutated *env* gene fragments was digested with both *Asp*718 (Boehringer Mannheim, Indianapolis, Ind.), which cleaves at the *Kpn*I site, and *Sal*I (Life Sciences). The recombinant 1.1-kb *env* fragments were purified by agarose gel electrophoresis and cloned into the equivalent site of pANV-A in place of the respective subgroup A *env* sequence. Recombinant pANV plasmids were propagated in *E. coli* DH1 or DH5.

DNA transfection and host range analysis. Approximately 5 μg of bacterial plasmid DNA containing the intact viral genome was transfected into C/O CEF cells by the Polybrene-dimethyl sulfoxide method (21) to generate individual virus stocks. Virus spread in the cell culture was monitored by assaying for the presence of virus-associated reverse transcriptase activity in the culture medium (36). Briefly, a sample from the culture supernatant was clarified in a microcentrifuge for 30 s and then a 15-μl sample was added to a 10-μl reaction mixture as described previously (5). The reaction was allowed to proceed for 2 h at 37°C, and the reaction products were spotted on DE81 paper, washed, and counted with a liquid scintillation counter. Reverse transcriptase activity values were normalized between assays to a standard virus sample. The standard values ranged from 20,000 to 60,000 cpm incorporated. Background incorporation (100 to 200 cpm) was determined from assays of the medium from uninfected cells maintained in parallel.

The host range of each recombinant virus was determined in U-251 MG and C/O CEF cells as follows. U-251 MG cells were plated at a density of 2×10^5 to 5×10^5 cells per 60-mm-diameter plate. C/O CEF cells were plated at a density of 2×10^5 to 5×10^5 cells per 60-mm plate. QT6 cells, which are resistant to subgroup B and D viruses, were added to the C/O CEF cells at a density of 1×10^4 to 5×10^4 cells per 60-mm plate to aid in the growth of the CEF cells at low density. On the following day, the cells were pretreated with 20 μg of Polybrene (37) in 1 ml of serum-free medium for 1 h. The medium was removed, and 1 ml of virus-containing medium was added. Cells were then incubated for 2 h at 37°C. The medium was removed and replaced with 2 ml of medium containing 200 μg of Geneticin (active form; G418; Life Sciences) per ml for the U-251 MG cell assay or 135 μg of G418 per ml for the C/O CEF assay. Mock-infected cells were maintained in parallel and remained negative for all assays. Cells were stained with crystal violet after 12 to 14 days of selection in G418. Virus titers (Fig. 1) are presented as numbers of cell colonies that would be obtained with 1 ml of a virus stock which incorporated 1,000 cpm/15 μl of virus-containing medium in the 25-μl reverse transcriptase assay. Actual virus stocks incorporated between 2.1×10^3 and 133×10^3 cpm in the assay. Experiments were designed to give between 10 and 200 colonies per plate. The absolute titers obtained with CEF cells are artificially low because of inefficient use of the simian virus 40 promoter to express the Neo^r-encoding gene (27a).

DNA sequence analysis. Recombinant M13 clones were sequenced by the dideoxy-chain termination procedure (31) as described by Bankier and Barrell (1). The nucleotide sequence of the entire *Kpn*I-to-*Sal*I fragment of each recombinant virus clone was determined on one strand by using single-stranded DNA as the template. Chemically synthesized oligonucleotides spanning the gp85-coding domain were used as primers.

Temperature sensitivity assay. A 2.5-ml volume of virus-containing medium was placed in polystyrene test tubes (12 by 75 mm) and either used immediately or stored at −70°C. The virus samples were thawed and placed in a 42 or 44°C water bath for various times. Cells were pretreated with Polybrene, and infection was performed at 37°C as described above. G418-resistant colonies were stained and counted. Individual assays were performed in duplicate.

RESULTS

Strategy for construction of recombinant viruses. We previously used sequence comparisons to identify five amino acid positions within the gp85-coding domain of the *env* gene that are unique to the subgroup D *env* gene sequence and, thus, are candidates for determinants of mammal tropism (6). Our strategy was to test the role of these specific amino acid residues along with larger segments of the subgroup D *env* sequence to define the determinants of the mammal tropism phenotype.

For these experiments, a set of replication-competent avian retrovirus vectors carrying a dominant selectable marker (Neo^r) were used. Viruses with different host range properties were constructed by exchanging the 1.1-kb *Kpn*I-to-*Sal*I restriction enzyme fragment encoding the N-terminal 85% of the gp85 portion of the *env* gene (and a small amount of the IN protein domain). This region has been shown previously to encode the major determinants of host range (5, 6, 12, 13). In this way, a subgroup B avian Neo^r virus (ANV-B8) and a subgroup D virus (ANV-D) were constructed from a subgroup A virus parent (Fig. 1; reference 6). The subgroup B *env* sequence was further modified by replacing portions of the *env* gene with sequences from the subgroup D *env* gene. Virus stocks were generated after transfection of the recombinant DNA genomes into CEF cells. Virus titers were determined on both CEF cells and the human cell line U-251 MG (to assess mammal tropism).

Growth and host range properties of recombinant viruses. To limit the region of the *env* gene to be considered, we first constructed a recombinant that brought the small *vr*1 and *vr*2 variable regions of the subgroup D *env* sequence into the subgroup B background (ANV-S23; Fig. 1). This recombinant was viable and had host range properties similar to those of the subgroup B virus. This observation narrowed the location of determinants important in mammal tropism to a 160-amino-acid stretch spanning variable regions *hr*1, *hr*2, and *vr*3 of gp85.

In the next set of recombinants, subgroup D-specific amino acid codons present in *hr*1 and *hr*2 were introduced into the subgroup B background. Oligonucleotide-directed mutagenesis was used to make the three subgroup D-specific amino acid changes in *hr*1 to yield ANV-BDHRIII, the two subgroup D-specific amino acid changes in *hr*2 (ANV-BDHRI), and the combination of these changes in *hr*1 and *hr*2 (ANV-134). All of these recombinants produced viable virus, and all had host range properties most similar to those of the subgroup B parent (Fig. 1). The two recombinants that included subgroup D-specific amino acids in *hr*1 (ANV-BDHRIII and ANV-134) showed slightly elevated infectivity for the human cells, but this level was still 50- to 100-fold below that seen with the subgroup D virus. This small increase in infectivity on human cells was seen for all recombinants with these three changes in *hr*1 (see below).

The next set of recombinant viruses contained an additional subgroup D-specific sequence from *hr*1 and *hr*2. Much of the sequence variability between subgroup B and D *env* genes is clustered in these regions, although the other sequence differences are not unique to either virus. ANV-R21 contained subgroup D sequences in *hr*1 and the upstream flanking region, ANV-P9 contained sequence changes in *hr*2, and ANV-S22 contained virtually all of the changes in *hr*1 and *hr*2 and included the additional change in the conserved region between *hr*1 and *hr*2. The ANV-P9 recombinant grew well as a virus and had a phenotype similar to that of the subgroup B parent. Both ANV-R21 and ANV-S22 grew poorly on chicken cells after transfection and showed low titers upon subsequent infection. Because of the slow growth, it is likely that the low titers are exaggerated because of loss of the selectable marker during the extended growth period (27a). What is striking is that the relative titers of these two viruses for human and chicken cells were significantly altered, showing an improvement in the ability to infect human cells relative to chicken cells (Fig. 1).

Finally, the role of sequence changes downstream of variable domains *hr*1 and *hr*2 was examined by incorporating changes found in the constant region between *hr*2 and *vr*3 (ANV-R51) or the changes in *vr*3 (ANV-BDVR3) into the subgroup B background. These changes, separately or together (ANV-R51DVR3), gave viable virus with growth and host range properties similar to those of the subgroup B virus. When these changes were included with the subgroup D-specific changes in *hr*1 (ANV-HR3R51 and ANV-HR3DVR3) or when the *vr*3 changes were included with the subgroup D-specific changes in *hr*1 and *hr*2 (ANV-134DVR3), viable virus with increased mammal tropism was obtained, although the extent of change in the host range was similar to that seen with the changes in *hr*1 alone (compare ANV-BDHRIII).

Construction of a novel mammal-tropic avian retrovirus. During oligonucleotide-directed mutagenesis of *hr*1 and *hr*2, we obtained one clone that had several additional sequence changes (ANV-4; Fig. 1). This clone is similar to ANV-134, which has the five subgroup D-specific codons in *hr*1 and *hr*2, except that one of the subgroup D-specific codons (proline 159) is replaced by a codon for another amino acid (to alanine) and two leucine codons are present in place of the proline and phenylalanine codons encoded by the subgroup D *env* gene (at positions 162 and 198, respectively). When a virus was reconstructed by using this DNA as the source of the *env* sequence, it was viable, although it had slightly reduced growth kinetics. Surprisingly, this virus showed significant tropism for mammalian cells, approaching that of the subgroup D parent (Fig. 1).

Mammal-tropic viruses have unstable Env proteins. The observation that several essentially random amino acid substitutions in the subgroup B *env* sequence could result in an Env protein that conferred enhanced mammal tropism suggested that this phenomenon is not associated with extended receptor specificity but rather is related to altered Env protein conformation. One manifestation of altered protein conformation can be the appearance of a temperature sensitivity phenotype. This possibility was tested by incubating ANV-B and ANV-D at 44°C for various times. At each time point, a virus sample was removed and its titer was determined on CEF cells. The results are shown in Fig. 2. Over a 4-h period, the subgroup B virus showed at most a twofold drop in titer. Over this same period, the subgroup D virus underwent a 2-order-of-magnitude drop in titer.

We next determined the temperature sensitivity of the recombinant viruses which showed various degrees of mammal tropism. In these experiments, the viruses were heated to 42°C for 3 h and then the remaining virus titer was determined on either CEF or U-251 MG cells. The four viruses that showed significant mammal tropism, ANV-D, ANV-4, and the two poorly replicating recombinants ANV-R21 and ANV-S22, were all significantly inactivated by incubation at the elevated temperature (Table 1). This was in contrast to the parental subgroup B virus and one of the non-mammal-tropic recombinant viruses (ANV-P9), which showed no significant sensitivity to heating. Several other viruses also showed changes in titer after heating. Two of the

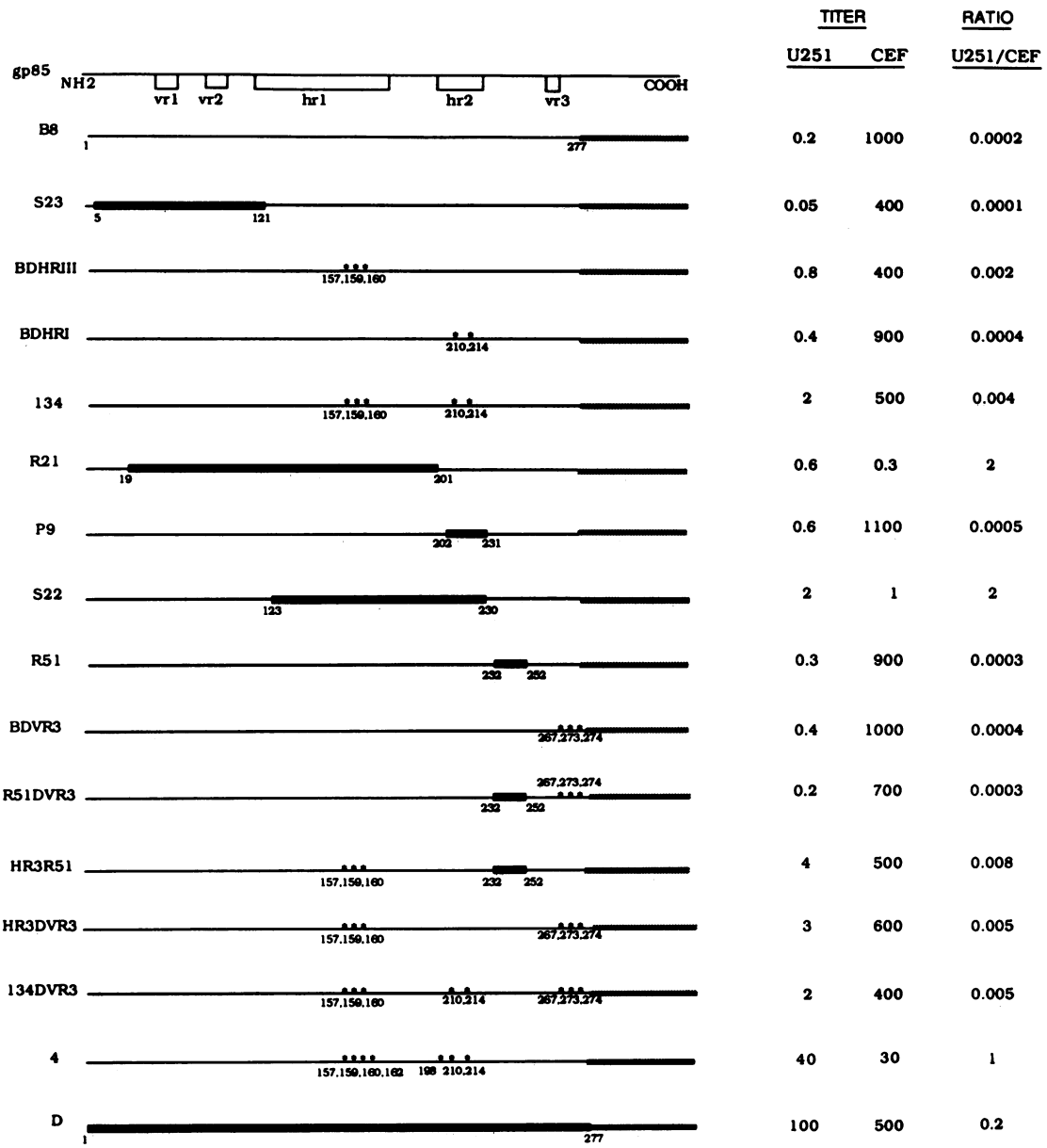

FIG. 1. Structures of recombinant ANV clones and virus titers in C/O CEF and U-251 MG cells. A linear schematic representation of the gp85 protein, from the amino terminus (NH_2) to the carboxyl terminus (COOH), is shown at the top. The structures of the recombinant virus clones are represented below. Variable domains *vr*1, *vr*2, *hr*1, *hr*2, and *vr*3, determined from comparisons of different subgroup virus sequences (5, 6, 12, 13), are shown at their approximate positions in the gp85 sequence. The hatched line to the right of *vr*3 indicates subgroup A *env* sequences from vector pANV-A. Heavy lines represent subgroup D sequences, and thin lines represent subgroup B sequences. For the recombinant viruses, the amino acid sequence positions that were switched to the subgroup D virus sequence are indicated beneath the line (6), as numbered from the amino terminus of SR-D RSV gp85. The asterisks show the locations of specific amino acid modifications produced by using mutagenic oligonucleotides. In the *hr*1 variable domain, the predicted amino acid sequences of the parental strains (6) that are relevant to the recombinants are L T N P P D N P F F (subgroup D amino acids 155 to 164) and L T D P G N N P F F (subgroup B amino acids 154 to 163). The sequence of ANV-4 in this region is L T N P A D N L F F. The relevant sequences in the *hr*2 variable region are N Y S I C E D V W (amino acids 209 to 217 for subgroup D) and N W S I C Q D V W (amino acids 209 to 217 for subgroup B). The titers (CFU) of the individual viruses for infection of chicken and human cells are given, as are the ratios of these titers. The titer represents the number of G418-resistant cell colonies obtained per milliliter of virus-containing medium by using a concentration of virus that gives 10^3 cpm incorporated when 15 μl of medium is added to the 25-μl reverse transcriptase assay. This was measured in an average of four experiments for each virus on U-251 MG cells and one to two experiments on CEF cells. Each measurement was done in duplicate. The final number is the average of the individual measurements rounded off to a whole digit. The extremes in standard deviation ranged from 100 ± 20 for ANV-D to 0.4 ± 0.5 for ANV-BDVR3.

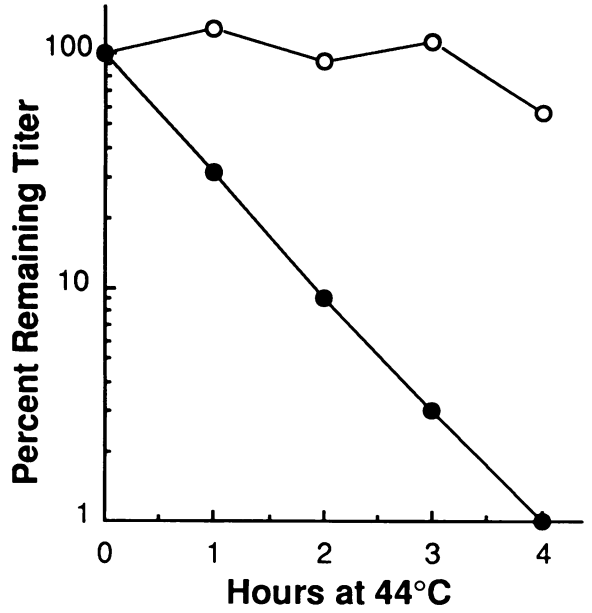

FIG. 2. Temperature sensitivity of ANV-D. Virus stocks of ANV-D (●) and ANV-B8 (○) were heated to 44°C. At the indicated times, samples were removed. The titers of all virus samples were determined on CEF cells. The values given are percentages of the original titers remaining after heating. The initial titer of ANV-D was 9.9×10^2 Neo^r colonies per ml of virus, and that of ANV-B8 was 4.5×10^3 Neo^r colonies per ml of virus.

recombinant viruses with the three subgroup D-specific amino acid substitutions in *hr*1 (ANV-BDHRIII and ANV-134) showed slightly enhanced infectivity for U-251 MG cells after heating at 42°C. However, the titers of these latter two viruses for their normal host (chicken cells) were not enhanced by heating (Table 1).

Analysis of other avian retroviruses. We extended the correlation between mammal tropism and temperature sensitivity by examining both of these parameters for a wider variety of avian retroviruses. ANV stocks were made from the SR-A parent, a RAV-1 recombinant that represented a second subgroup A virus, a Prague C RSV recombinant, and a RAV-0 recombinant representing a subgroup E virus (5, 6).

TABLE 1. Temperature sensitivity of subgroup B and D *env* gene recombinants

Virus[a]	% of titer remaining[b]						
	C/O CEF cells			U-251 MG cells			
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 4
ANV-B8	85			74	110		
ANV-P9	120	73	83	100	97	100	
ANV-BDHRIII	86	81	74	120	190	140	
ANV-134	83	93		78	200	170	
ANV-R21				31	16		
ANV-S22				47	47	37	
ANV-4	32	21	19	40	35	19	19
ANV-D	27	17	2	21	21	12	21

[a] Avian Neo^r viruses (Fig. 1).
[b] G418-resistant cell colonies after infection with a virus stock that had been heated to 42°C for 3 h. Expressed as a percentage compared with untreated virus from the same virus stock.

TABLE 2. Summary of temperature sensitivity and mammal tropism host range properties of different subgroup viruses

Virus	Source of gp85	Subgroup	Temperature sensitivity[a]		Mammal tropism[b]
			Avian cells	U-251 MG cells	
ANV-A	SR-A RSV	A	71		0.002
ANV-A1	RAV-1	A	76		0.0006
ANV-B8	RAV-2	B	113	92	0.0002
ANV-C	Prague C RSV	C	48		0.0003
B77	B77 RSV	C	74	5	0.005
ANV-D	SR-D RSV	D	3	19	0.2
ANV-E	RAV-0	E	86		0.00004

[a] Temperature sensitivity was assessed at either 44 or 42°C (subgroup B and D viruses on U-251 MG cells) for 3 h. The values given are percentages of infectivity remaining after heat treatment. The data for ANV-B8 and ANV-D are from Fig. 2 on CEF cells and Table 1 on U-251 MG cells. Subgroup A, C, and E viruses were assayed on QT6 cells.
[b] Ratio of unheated-virus titer on U-251 MG cells to that on avian cells. Data for ANV-B8 and ANV-D are from Fig. 1. The values given are averages of two experiments which all varied by less than twofold, except for ANV-E, which, because of its low titer on U-251 MG cells, had greater variability.

In addition, the B77 subgroup C RSV was used to rescue an *env*-defective virus with a Neo^r-encoding insert. Of these viruses, only the B77 strain of RSV has been shown to be mammal tropic (4, 14), although the SR-A version of ANV did show a low level of infectivity on certain mammalian cells (6). Each virus stock was tested for the ratio of infectivity on avian cells versus that on the human cell line (Table 2). ANV-D was the most mammal-tropic virus, approximately 40-fold more so than the other mammal-tropic virus, B77. All of the other viruses showed less infectivity when mammalian cells were used, i.e., between 2.5- and 100-fold less relative to B77.

Two of the viruses in this group were temperature sensitive for infectivity. ANV-D displayed this phenotype when assayed on both avian cells and mammalian cells (Table 2). The B77 virus also showed a temperature sensitivity phenotype but, surprisingly, only when assayed on the mammalian cell line.

DISCUSSION

Growth properties of the recombinant viruses. Most of the recombinant viruses grew well, as predicted, since the two parental viruses use the same receptor on chicken cells and have extensive sequence similarity within the gp85-coding domain (6, 14). However, two of the recombinants (ANV-R21 and ANV-S22) grew poorly. Both of these recombinants contained subgroup D sequences spanning the *hr*1 variable domain and included subgroup D-specific sequences either upstream or downstream of *hr*1 (Fig. 1). We assume that the reduced growth is the result of either inefficient localization of the chimeric glycoprotein to the virus because of poor folding, poor interaction with the host cell receptor, or reduced ability to promote membrane fusion, although these possibilities were not examined directly. We considered the possibility that variable region *vr*3, which can have an indirect role in receptor recognition (12), might be able to revert the poor replication properties of these two viruses. Two additional virus constructs that introduced the subgroup D sequence into the *vr*3 region of ANV-R21 and ANV-S22 were made. However, these viruses also had low titers on C/O CEF cells (data not shown). Apparently, amino

acids present in the subgroup D sequence of *hr*1 require some specific but unidentified interaction outside of this region to be fully functional.

Mammal tropism determinants of SR-D RSV. We examined 13 recombinants between the non-mammal-tropic subgroup B virus and the mammal-tropic subgroup D virus. Our strategy was to isolate the five variable regions in making the recombinants to assess their individual contributions to the mammal tropism phenotype. For four of the variable regions (*vr*1, *vr*2, *hr*2, and *vr*3), there was no contribution to a mammal tropism phenotype when they were placed into the background of the non-mammal-tropic parent (Fig. 1). By contrast, every change tested in the *hr*1 variable region affected the host range properties and temperature stability of the recombinant virus, although none of the recombinants completely duplicated the host range and growth characteristics of the parental subgroup D virus.

We have previously suggested that five subgroup D-specific amino acids found in *hr*1 and *hr*2 might be involved in conferring a mammal tropism host range (6). The two changes in *hr*2 had no effect on this phenotype (ANV-BDHRI; Fig. 1). However, the three subgroup D-specific amino acids in *hr*1 consistently had a modest effect on the host range (ANV-BDHRIII, ANV-134, ANV-HR3R51, ANV-HR3DVR3, and ANV-134DVR3; Fig. 1). While the level of infectivity on mammalian cells increased beyond that of the non-mammal-tropic subgroup B parent, it was still less than 5% of that seen with the subgroup D parent and addition of other small changes seen in the subgroup D sequence did not augment the mammal tropism phenotype (Fig. 1).

Two recombinants contained virtually all of the subgroup D *hr*1 sequence plus either an upstream or downstream flanking sequence (ANV-R21 and ANV-S22; Fig. 1). As noted above, both of these recombinants grew poorly. Nevertheless, the resulting viruses showed significant infectivity on mammalian cells relative to chicken cells, also implicating *hr*1 as an important determinant of mammal tropism.

Finally, one DNA clone that had fortuitously incorporated two substitutions in *hr*1 and one substitution in the constant region between *hr*1 and *hr*2 during the mutagenesis procedure gave rise to virus that had reasonable growth properties in chicken cells and a mammal tropism phenotype (ANV-4; Fig. 1). The phenotype of this virus suggests that mammal tropism is the result not of altered receptor specificity (allowing interaction with a putative receptor on mammalian cells) but rather of sequence variation within a region important for glycoprotein function.

Mammal tropism correlates with temperature sensitivity. Each of the viruses with alterations in *hr*1 had both an increase in infectivity on mammalian cells and a temperature sensitivity phenotype for infectivity, although the response to temperature was not the same for all of the recombinants. At one extreme, recombinants with the three subgroup D-specific amino acid substitutions in *hr*1 (two were tested, ANV-BDHRIII and ANV-134) showed small but consistent increases in titer on mammalian cells after heating to 42°C (Table 1). This increase in titer was not seen on chicken cells. Thus, heating seemed to increase the abilities of these viruses to penetrate heterologous cells while not affecting their abilities to interact with the normal host receptor.

The second type of temperature sensitivity was seen with the viruses that had the more extensive mammal tropism phenotype (ANV-D, ANV-4, ANV-R21, and ANV-S22). All of these viruses were inactivated by heating to a much greater extent than either the non-mammal-tropic subgroup B virus or a virus, ANV-P9, with an alteration outside of the *hr*1 variable region (Fig. 2; Table 1). These results suggest that the viral glycoproteins associated with the mammal-tropic viruses are in unstable conformations and, therefore, are more easily inactivated by heating.

We extended the correlation between mammal tropism and temperature sensitivity by examining a number of avian retroviruses, including another mammal-tropic avian retrovirus, the B77 strain of RSV, a subgroup C virus (4, 14). We used B77 RSV to rescue a replication-defective viral genome containing the gene for Neo^r. We found that the rescued virus containing the gene for Neo^r was temperature sensitive in infectivity of human cells (Table 2). Since all of the viral proteins were donated by B77 RSV in this experiment, we cannot localize the temperature sensitivity phenotype to a specific viral gene product. However, the surprising, and unexplained, observation that the appearance of the temperature sensitivity phenotype is host cell dependent suggests a host range phenomenon most likely involving the viral glycoprotein. No other virus tested showed either temperature sensitivity or a mammal tropism phenotype equivalent to that of either B77 RSV or ANV-D (Table 2).

Alternative pathway of virus penetration. Studies of the hemagglutinin glycoprotein of influenza virus suggest that conformational changes in the glycoprotein complex are important for fusion (44). The conformational changes appear to be necessary to expose a hydrophobic segment of the transmembrane protein (the fusion peptide). While fusion normally occurs in the acidic environment of endosomes, temperature-sensitive hemagglutinin protein mutants have been shown to fuse to liposomes at a higher pH (30). These data suggest that the mutant glycoproteins are conformationally unstable and denature readily at an elevated temperature. As a result of this instability, the mutant glycoprotein complex can fuse in a less acidic environment.

By analogy, the instability of the subgroup D virus may result in a more fusogenic virus. For viruses that fuse at the plasma membrane surface, a high-affinity receptor may be required such that binding to the receptor induces the conformational change needed to initiate fusion. A conformationally unstable glycoprotein complex could fuse without binding to a high-affinity receptor on the cell surface. The subgroup D ASLV would therefore maintain affinity for its normal receptor (the *tv-b* gene product), while the instability of the glycoprotein complex would enable fusion to occur in the absence of the normal receptor. Fusion in the absence of a normal receptor could account for the ability of subgroup D viruses to infect chicken cells lacking the *tv-b* locus (14) and their ability to abrogate partially the effects of interference by a subgroup B virus (2). In the absence of a high-affinity receptor, attachment must be mediated by other mechanisms. The variability of titer of the subgroup D virus on different cell types (6) suggests that the ability of the virus to attach varies with the cell type.

Implications of glycoprotein instability. Our experiments suggest that instability in the viral surface glycoprotein could account for the mammal tropism seen with certain avian retroviruses of the ASLV group, such as SR-D and B77 RSV. A similar mechanism could also account for the ability of human immunodeficiency virus type 1 to infect cells through a pathway that does not include its receptor, CD4 (7, 8, 16, 23, 24, 35).

Envelope glycoproteins of viruses frequently have the most sequence diversity of all viral proteins. This is presumed to be the result of immune selection for antigenic

variants. The resulting amino acid substitutions may not be neutral for glycoprotein function, and the preponderance of substitutions that have an effect on function will have an adverse effect. Thus, in any population of viruses that is undergoing immune selection there is a possibility of finding variants with altered glycoprotein function. Mammal-tropic strains of the ASLV group may be one example of such variants.

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