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Selection of an Avian Retrovirus Mutant with Extended Receptor Usage

RANDY A. TAPLITZ[1]† AND JOHN M. COFFIN[2]*

Division of Infectious Diseases, Department of Medicine, Tufts University/New England Medical Center,[1] and Department of Molecular Biology and Microbiology, Tufts University School of Medicine,[2] Boston, Massachusetts 02111

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Receptor recognition by avian retroviruses is thought to involve the interaction of two regions of the SU protein, *hr*1 and *hr*2, with the host cell surface receptor. These regions exhibit considerable variation, concordant with differences in receptor usage among the many avian leukosis virus subgroups. We hypothesize that some retroviruses have altered receptor usage in response to selective pressures imposed by receptor polymorphisms in their hosts. To test this hypothesis, we passaged td-Pr-RSV-B on cocultured permissive chicken (C/E) and nonpermissive quail (QT6/BD) cells. A variant virus with an expanded host range was identified at passage 29 and ultimately shown to be identical in sequence to td-Pr-RSV-B, except for changes at codons 155 and 156 of SU amino acid corresponding to two amino acid changes within *hr*1. Superinfection resistance studies suggest that the variant virus recognizes the subgroup B receptor on chicken cells and the subgroup E receptor on quail cells. These findings indicate that altered receptor usage can be conferred by small changes in *env* and may point to a key region for receptor interaction. Further, they demonstrate the evolutionary potential of retroviral *env* genes to alter receptor usage in response to appropriate selective pressure.

The *env* gene of avian retroviruses encodes two glycoproteins, SU (gp85), the surface subunit, and TM (gp37), the transmembrane subunit. These proteins are involved in specific recognition and binding of cell surface receptors, as well as penetration of the host cell membrane. Additionally, they induce superinfection resistance by blocking interaction of the virus with cellular receptors (12, 19, 20).

The cell surface receptors for seven retroviruses have been described; these include human immunodeficiency virus (CD4), ecotropic murine leukemia virus (mCAT-1), gibbon ape leukemia virus (hPiT-1), amphotropic murine leukemia virus (RAM-1), bovine leukemia virus, and Rous sarcoma virus (RSV) subgroups A and B (Tva and CAR1) (2–4, 8, 25, 26, 37). Although the structures and natural functions of these receptor proteins are very different from each other, the basic organizational structures of the Env glycoproteins from the different retroviruses recognizing these receptors are grossly similar (5, 22–24). This similarity supports the speculation of similarity in receptor binding among disparate retroviruses.

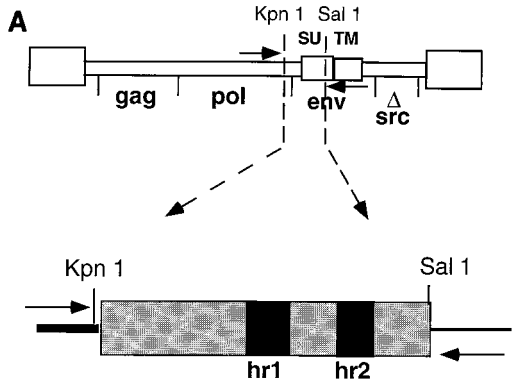
Avian retroviruses are useful in the study of molecular mechanisms important in host range and receptor recognition because they display a remarkable diversity of receptor utilization within a group of otherwise very closely related viruses. The best studied of these viruses can be divided into five subgroups, A through E, based on (i) the ability to infect genetically defined avian cells, (ii) interference patterns, and (iii) neutralizing antibody cross-reaction. Viruses of five other subgroups, F to J, have been isolated from avian species as well (38).

In chickens, three autosomal loci (Tv-a, Tv-b, and Tv-c) controlling cell susceptibility to infection by viruses of different subgroups have been defined. Viruses of subgroup A, and most likely those of subgroups B and C, recognize independent receptors that are directly encoded by the corresponding loci (4, 16, 39). Viruses of subgroups B, D, and E recognize closely related, probably allelic receptors (38). B and D viruses are apparently identical in receptor usage, although their SU protein sequences differ somewhat. In chickens, resistance to subgroup B viruses is associated with resistance to subgroup E viruses, and preinfection of chicken cells with subgroup B or D virus prevents superinfection by subgroup E virus. This interference is not reciprocal in chickens, since susceptibility to subgroup E virus is always associated with susceptibility to subgroup B and D viruses. Other avian species do not exhibit patterns of resistance identical to that of chickens; for example, turkey and quail embryo fibroblasts are susceptible to infection by subgroup E virus but resistant to infection by subgroup B and D viruses (12, 21, 36, 38).

The region of the viral genome that determines subgroup specificity was initially determined by T_1 oligonucleotide mapping and nucleotide sequencing to reside in the middle third of the SU protein (15) (Fig. 1A). In this region, several domains with low amino acid identity among the subgroups exist within a highly conserved framework (Fig. 1B). The presence, within the variable regions, of conserved amino acids such as proline, glycine, and cysteine and hydrophobic amino acids, which could play a key role in protein folding, implies similar overall structures of this region in the different subgroups. It is possible that the regions of heterogeneity extend to the surface of the protein and interact with binding sites on the host cell receptor. Analysis of the *env* gene of NTRE-4, a recombinant between subgroup B td-PR-RSV-B and RAV-0, a subgroup E virus that can infect both C/E and T/BD cells, revealed a composite structure, with an *hr*1 region derived from the subgroup B virus and *hr*2 of subgroup E (15, 34). This organization suggested that two variable regions of different subgroups could interact to recognize both types of receptor. Analysis of rescued virus from molecular clones carrying various combinations of these variable regions from different subgroup vi-

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111. Phone: (617) 636-6528. Fax: (617) 636-8086. E-mail: Jcoffin_par@opal.tufts.edu.

† Present address: Division of Infectious Diseases, Department of Medicine, University of California, San Francisco, CA 94143.



B

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                                                    50
RAV-1      DVHLLEQPGNLWITWASRTGQTDFCLSTQSATSPFQTCLIGIPSPISEGDFKGYVSDTNCATSGTD
PR-B       DVHLLEQPGNLWITWANRTGQTDFCLSTQSATSPFQTCLVGVPSPISEGDFKGYVSD-NCTTVGTH
PR-C       DVHLLEQPGNLWITWANRTGQTDFCLSTQSATSPFQTCLIGIPSPISEGDFKGYVSDTNCSTVGTD
SR-D       DVHLLEQPGNLWITWAMRTGQTDFCLSTQSATSPFQTCLVGIPSPISEGDFKGYVSDTNCTTVGTH
RAV-0      DVHLLEQPGNLWITWANRTGQTDFCLSTQSATSPFQTCLIGIPSPISEGDFKGYVSDTNCTTLGTD

CONSENSUS  DVHLLEQPGNLWITWANRTGQTDFCLSTQSATSPFQTCLIGIPSPISEFDFKGYVSDTNCTTVGTD

                                                   100
RAV-1      RLVSSADFTGGPDNSTTLTYRKVSCLLLKLNVSMWDEPPELQLLGSQSLPNITNITQISGIVGGCV
PR-B       RLVSS-GIPDGPDNSTTLTYRKVSCLLLKLNVSLLDEPSELQLLGSQSLPNITNITRIPSVAGGCI
PR-C       RLVLSASITGGPDNSTTLTYRKVSCLLLKLNVSMWDEPPELQLLGSQSLPNVTNITQVSGVAGGCV
SR-D       RLVSS-GIPGGPDNSTTLTYRKVSCLLLKLNVSMWDEPPELQLLGSQSLPNIANITQIPGVAGGCI
RAV-0      RLVSSASITGGPDNSTTLTYRKVSCLLLKLNVSMWDEPPELQLLGSQSLPNITNITQISGVTGGCV

CONSENSUS  RLVSSA-ITGGPDNSTTLTYRKVSCLLLKLNVSMWDEPPELQLLGSQSLPNITNITQISGVAGGCV

                                  150 hr1
RAV-1      GFRPKGVPWY-LGWSRQEATRFLLRRP-------SFSNSSKPFTVVTADRHNLFYGSEYCGAYGYR
PR-B       GFTPYGSPAGVYGWDRRQVTHILLTDPGNNPFFDKASNSSKPFTVVTADRHNLFMGSEYCGAYGYR
PR-C       YFAP-RATGLPLGWSKQGLSRFLLRHP-----FTSTSNSTEPFTVVTADRHNLFMGSEYCGAYGYR
SR-D       GFTPYGSPAGVYGWGREEVTHILLTNPPDNPFFNRASNSTEPFTVVTADRHNLFMGSEYCGAYGYR
RAV-0      GFAPHSNPSGVYGWGRRQVTHNFLIAPWVNPFFNSASNSTEPFTVVTADRHNLFMGSEYCGAYGYR

CONSENSUS  GF-P-G-P-GVYGW-R--VTH-LL--P--NPFFNSASNSTEPFTVVTADRHNLFMGSEYCGAYGYR

      200                  hr2                          250
RAV-1      FWNIYNCSQ-VGQQ--YRCGNARRPRPGHPETQCTRR-GGKWVNQSRKINETEPFSFTVTCTASNL
PR-B       FWEMYNCSQMRQN--WSICQ--DVWGRGPPESWCTST-GGIWVNQSKAINETEPFSFTANCTGSNL
PR-C       FWEIYNCSQTR-N--TYRCG--DVGGTGLPETWC-RGKGGIWVNQSKEINETEPFSFTANCTGSNL
SR-D       FWEMYNCSQIR-N--YSICE--DVWGPGLPESWCART-GGTWVNKSKEINETEPISFTVNCTGSNL
RAV-0      FWEIYNCSHRFDNFDIYTCG--DVQTVKSPEKQCVGG-GGIWVNQSKEINETEPFSFTANCTASNL

CONSENSUS  FWEIYNCSQ-R-N---Y-CG--DV-G-G-PE-WC-R--GGIWVNQSKEINETEPFSFTANCTGSNL

                                  300
RAV-1      GNVSGCCGKAGMILPLGAWIDSTQGSFTKPKALPPAIFLICGDRAWQGIPSRPVGGPCYLGKLTML
PR-B       GNVSGCCGEPITILPPGAWVDSTQGSFTKPKALPPAIFLICGDRAWQGIPSRPVGGPCYLGKLTML
PR-C       GNVSGCCGEPITILPLGAWIDSTQGSFTKPKALPPAIFLICGDRAWQGIPSRPVGGPCYLGKLTML
SR-D       GNVSGCCGEAITILPPGAWVDSTQGSFTKPKALPPGIFLICGDRAWQGIPSRPVGGPCYLGKLTML
RAV-0      GNVSGCCGKTITILPSGAWVDSTQGSFTKPKALPPAIFLICGDRAWQGIPSRPVGGPCYLGKLTML

CONSENSUS  GNVSGCCGE-ITILPPGAWVDSTQGSFTKPKALPPAIFLICGDRAWQGIPSRPVGGPCYLGKLTML

                                  350
RAV-1      APKHTDILKVLVNSSQTGIRRKR
PR-B       APNHTDILKILANSSRTGIRRKR
PR-C       APNHTDILKILANSSRTGIRRKR
SR-D       APNHTDILKILANSSRTGIRRKR
RAV-0      APNHTDILKVLANSSRTGIRRKR

CONSENSUS  APNHTDILKILANSSRTGIRRKR
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FIG. 1. (A) Structure of the td-PR-RSV-B provirus. Shown schematically are the *env* gene, the *Kpn*I site in *pol* and the *Sal*I site in *env* used to reconstitute the virus, and the locations of *hr*1 and *hr*2 within the fragment. The arrows represent PCR primers used to amplify the *hr*1-*hr*2 region for cloning. (B) Amino acid sequences of the SU region of *env* from ALV subgroups A to E. The predicted sequences of the mature form of SU from subgroups A to E are compared. A consensus (defined as the same amino acid at a given position in at least three of the six viruses) sequence is also shown. *hr*1 and *hr*2 are boxed. The data are from references 6, 15, 15a, and 30.

ruses suggested that receptor binding is determined principally by the interaction of the two major variable regions, *hr*1 and *hr*2, in the middle of the SU protein (6, 14, 20).

Sequence analysis of the different avian retrovirus *env* genes suggests that the variable regions evolved from a common ancestor. It is likely that the ability of retroviruses to evolve rapidly has allowed them to acquire the ability to use different receptors in response to the development of genetic resistance in the host. This host range expansion by these avian retroviruses likely occurred by both point mutation and exchange of *hr* regions by recombination (11, 15a).

Although the sequence differences among viruses with different receptor specificities are fairly large, it is likely that they have evolved in small steps that would be possible to replicate in simple cell culture systems. To test this hypothesis and to delineate specific sequences important for host range variation, we have devised an experimental system for selection of evolutionary variants in which selective pressure for an expanded host range is exerted at a sufficiently modest level to allow the occurrence and subsequent selection of rare host range variants. A variant virus with an altered host range was selected by using this method and found to possess two amino acid changes in Env. These changes confer an extended host range on a subgroup B virus, allowing it to infect QT6/BD cells by using the subgroup E receptor. These selected mutations may point to a key region for receptor interaction in the Env protein. Further, these findings demonstrate the evolutionary potential of retroviral *env* genes to alter receptor usage in response to appropriate selective pressure.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts (C/E; C300) with no endogenous virus were prepared from fertilized eggs (USDA Poultry Station, East Lansing, Mich.). QT6 cells (QT6/BD) are a continuous line derived from a methylcholanthrene-induced fibrosarcoma of Japanese quail (28). QT6lac914 cells are QT6 cells which have been stably transfected with the bacterial β-galactosidase (*lacZ*) gene in an avian leukosis virus (ALV)-based vector (9, 29, 32). The *lacZ* vector is rescuable by superinfection with ALV and can yield titers measurable by a lac^+ colony-forming assay of up to 10^7 CFU/ml (our unpublished data). The molecular clone of the transformation-defective deletion mutant td-PR-RSV-B is a full-length virus permuted into PBR322 at the *Sal*I site and has been extensively characterized in this laboratory (15). The RAV-1 (31), Prague-RSV-E, and NTRE-4 (15, 34) viruses have been previously characterized in our laboratory. All viruses were rescued from molecular clones and grown on cells free of closely related endogenous viruses. Cells were maintained in modified Richter's medium with 10% fetal calf serum.

Transfection of cloned viral DNA and determination of virus titer. Permuted viral inserts were excised from vector DNA by *Sal*I digestion and ligated by using T4 DNA ligase. Avian cells were transfected by the DEAE-dextran method as previously described (13) or with the lipofectamine reagent as recommended by the manufacturer (Gibco BRL). Production of infectious virus was detected by testing for reverse transcriptase (RT) in the culture medium by using 1 ml of sedimented culture medium incubated with 25 ml of assay buffer (50 mM TrisCl [pH 7.5], 60 mM NaCl, 10 mM $MgCl_2$, 20 mM dithiothreitol, 10 mM dTTP, 5 mg of oligo(dT) per ml, 10 mg of poly(RA) per ml, 0.05% Nonidet P-40, 0.2 mCi of [^{35}S]TTP) at 37°C for 1 h. This reaction mixture was then filtered through Whatman DEAE paper and washed three times with 6% trichloroacetic acid, rinsed with 95% ethanol, added to 7 ml of scintillation fluid, and counted.

Measurement of the titer of the virus passed on the QT6lac914 cell line was also performed by determination of β-galactosidase expression. Briefly, 48 to 72 h after infection, monolayers were fixed with 2% paraformaldehyde–0.2% glutaraldehyde–0.1 M $NaPO_4$. Cells were then washed with phosphate-buffered saline and stained overnight with 0.1 M $NaPO_4$ (pH 7.3)–1.3 mM $MgCl_2$–3 mM $K_3Fe(CN)_6$–3 mM $K_4Fe(CN)_6$–1.0 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; U.S. Biochemical, Cleveland, Ohio) per ml.

Infections. One milliliter of a viral stock or infected cell supernatant filtered through a 0.45-μm-pore-size Millipore filter was used to infect monolayers of cells 24 h after plating in the presence of 1.5 μg of Polybrene. Infections were incubated for 45 min at 37°C, and rocked every 15 min.

Determination of viral sequences within cellular DNA. To confirm the absence of endogenous virus in C300 cells, Southern blot analysis was performed by using a whole ALV probe ^{32}P labeled by nick translation. To demonstrate the presence of exogenous envelope sequences in previously nonpermissive cells, we performed Southern blot analysis by using a whole ALV probe and PCR amplification. To detect *env* sequences, DNA was amplified by using the following primers: oligonucleotide pol (nucleotides 4964 to 4987, based on the PR-RSV-C sequence [31], 5′ to the *Kpn*I site in the *pol* gene), 5′ TGG AAA AAC AGG GAA CAC TGA TAA G 3′; oligonucleotide sal (nucleotides 6061 to 6083 3′ to the unique *Sal*I site in *env*), 5′ GCT GTC ATG CGT TCC TTC AAA AT 3′.

Subcloning of the variant *env* fragment into the wild-type molecular clone of td-PR-RSV-B. Wild-type td-PR-RSV-B cloned DNA permuted into PBR322 at the *Sal*I site at nucleotide 6059 near the SU-TM junction was digested with *Kpn*I and partially digested with *Sal*I such that a differential pattern of *Sal*I digestion was generated, as two *Sal*I sites exist in the construct. This allowed us to isolate the fragment containing the whole construct minus the 1.1-kb *env* fragment, which was gel purified and ligated to a similarly digested mutant 1.1-kb *env* fragment by using T4 DNA ligase. The identity of the recombinant mutant clone was verified by restriction enzyme digestion and nucleotide sequence analysis.

Nucleotide sequence analysis. DNA sequence analysis was performed on cloned or genomic DNA by using the dideoxynucleotide chain termination method. Additionally, direct sequencing of purified PCR products was performed by thermal cycle sequencing with $Vent_R$ (exo-) DNA polymerase (New England BioLabs, Beverly, Mass.).

RESULTS

Strategy used to select for a host range variant virus. Despite the variety of receptor utilization among avian retroviruses, mutations conferring altered receptor usage have not been previously observed, suggesting the necessity for multiple simultaneous changes. We hypothesized that we could select such a rare variant by infecting cells with a virus under moderate selective conditions, avoiding population bottlenecks that would limit diversity (10, 11, 17, 18). With repeated passage under these conditions, a rare mutant that confers a selective advantage should become the predominant virus in the population. Thus, if a virus were passaged on cells under conditions in which it would be advantageous but not essential to have an extended host range, a mutant with an extended host range might be selected. Such selection could be accomplished by serially passaging a virus on mixtures of two types of cells, one permissive for infection and one nonpermissive due to lack of a suitable receptor. A variant virus with an extended host range would be selected on the basis of the greater number of available target cells.

To test this strategy, we established a cocultivation system in which a subgroup B ALV (td-Pr-RSV-B) was passaged on a mixture of susceptible C300 (C/E, ev^-) and resistant (QT6/BD) cells (Fig. 2). To ensure that recombinants with endogenous proviruses providing an extended host range (34) could not occur, we confirmed the absence of ALV-like endogenous proviruses in the C300 cells (data not shown). Undiluted supernatant from infected cultures was passaged repeatedly on fresh cocultures. We anticipated that a variant virus able to infect QT6 cells would eventually appear in the virus population. To test for the presence of such a virus, supernatant was used to infect QT6lac914 cells, which contain an ALV-based *lacZ* vector (32). A virus with an altered host range could then be identified by its ability to rescue the *lacZ* gene into infectious virus, which could be tested for by subsequent infection and staining of C300 or QT6 cells, as well as by RT assays.

We were unable to detect a virus capable of infecting nonpermissive QT6lac914 cells in the early passages on the cell mixture. However, assays of supernatant from QT6lac914 cells infected with undiluted virus from viral passages 28 and 29 revealed an increase in RT activity over the baseline, consistent with the appearance of a variant virus able to infect the previously nonpermissive cells (Fig. 3A). Additionally, the *lacZ* gene was transferred with low efficiency to chicken cells by infection with this supernatant, again suggesting the presence of a variant virus with an altered host range (data not shown). Southern blot analysis of genomic DNA from QT6 cells infected with supernatant from viral passages 1, 28, and 32 revealed the presence of the expected Pr-RSV-B virus-specific bands in the QT6 cells infected with virus from viral passages 28 and 32, but not 1 (data not shown), confirming the newly acquired ability to infect these cells.

To further enrich for virus capable of infecting QT6lac914 cells, we created a new cell line, QT6lac914/PR-B(40), by infecting QT6lac914 cells with supernatant from the cocultivations at viral passage 40 and serially passaging these cells. These cells initially produced only small amounts of virus, but on subsequent passages, the virus from this cell line grew to a high titer (Fig. 3B) and was able to transfer the *lacZ* gene to both C/E and QT6 cells with high efficiency (data not shown).

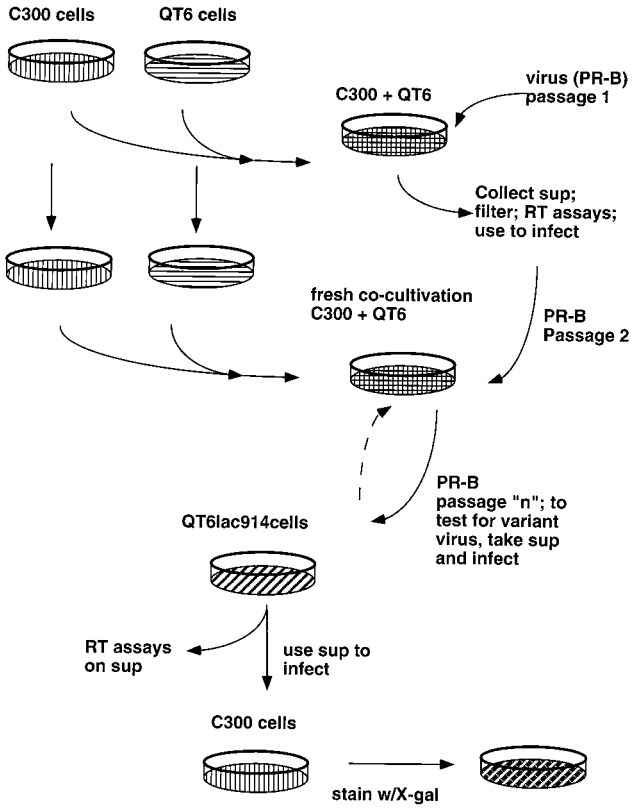

FIG. 2. Experimental scheme showing serial virus (td-PR-RSV-B, Pr-B) passage on cocultures of permissive chicken cells (C300) and nonpermissive quail cells (QT6). At each passage, supernatants (sup) are tested for an extended host range by determining their ability to infect and rescue a *lacZ* vector from QT6 cells.

Mutations conferring an extended host range. We considered it most probable that any mutation conferring an extended host range would lie within the central region of SU encompassing *hr*1 and *hr*2 (Fig. 1). Therefore, a 1.1-kb fragment of the *env* gene including this region was PCR amplified from C300 cells infected with C300/QT6 viral passage 32, from QT6 cells infected with C300/QT6 viral passages 28 and 32, and also from QT6lac914/PR-B(40) cells infected with viral passages 9, 11, and 30. The fragment pattern of these PCR products upon digestion with *Eco*RI and *Bam*HI was consistent with subgroup B virus and excluded the possibility of contamination with another ALV strain (data not shown). These amplification products were subsequently cloned and sequenced. The sequences of the viruses from six coculture clones (C300/QT6 viral passage 32 grown on chicken cells and passages 28 and 32 grown on QT6 cells) demonstrated only the wild-type sequence through *hr*1 and *hr*2. However, four different clones from the QT6lac914/PR-B(40) cells at passages 9, 11, and 30 showed the same two sequence changes. These changes, at codons 155 and 156 of *env*, result in two amino acid changes in the predicted protein product (Fig. 4).

To determine whether the two mutations were sufficient to confer the extended-host-range phenotype, we exchanged the 1.1-kb fragment from an intact virus containing the mutant *env* gene (S20) into the wild-type molecular clone of td-PR-RSV-B (Fig. 1A). The particular clone used for this subcloning also showed two synonymous nucleotide changes in *env,* at nucleotides 3 and 571 (Fig. 4). The latter change was seen only in

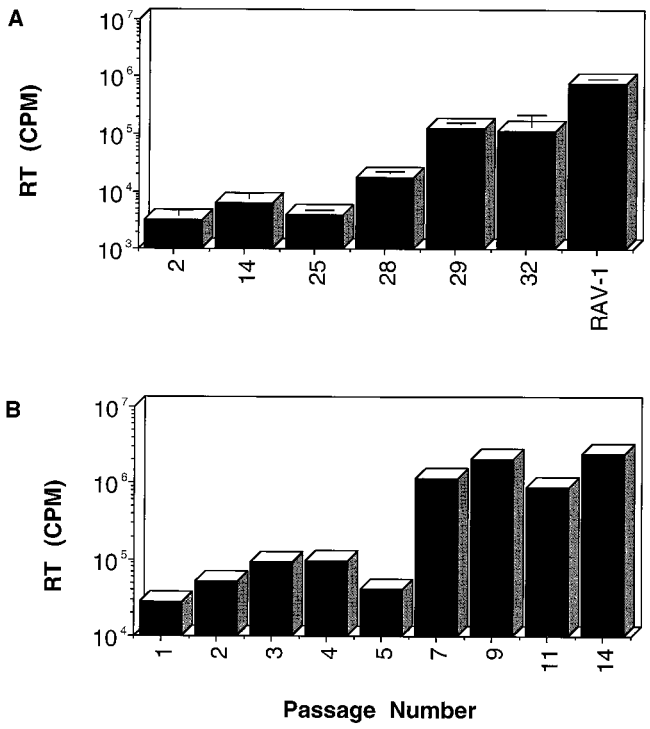

FIG. 3. Appearance of a virus capable of infecting quail cells. td-PR-RSV-B was serially passaged on C300-QT6 cell mixtures as described in the text. (A) At the indicated passage numbers, supernatant was used to infect QT6lac914 cells. (B) The QT6lac914/PR-B(40) cell line was created by infecting QT6lac914 cells with supernatant from cocultivations at viral passage 40, as described in the text. RT assays were then performed on the supernatant. QT6lac914 cells are permissive to RAV-1 infection, which was used as a positive control. The background in this assay was about 5,000 cpm.

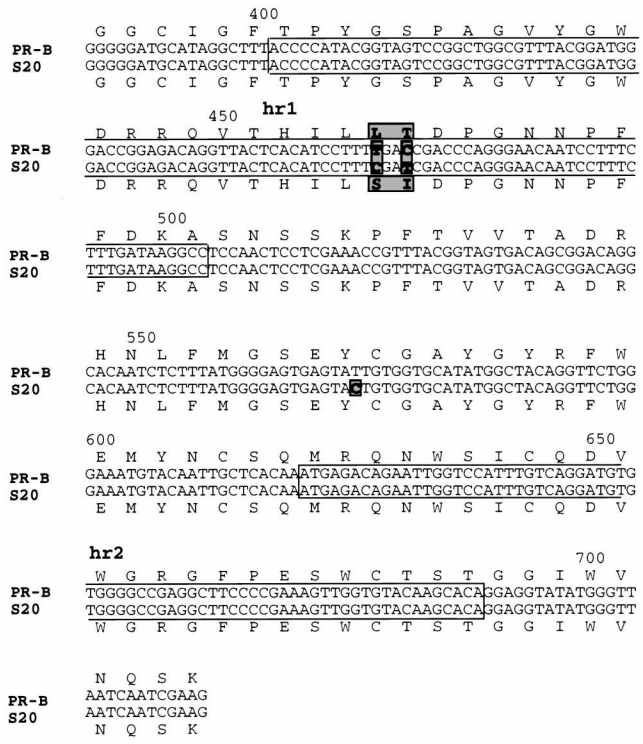

FIG. 4. Comparison of the nucleotide and amino acid sequences of a portion of the 1.1-kb fragment of *env* from the viral variant with an extended host range and wild-type td-PR-RSV-B. The sequence is numbered from the start of SU.

this particular clone, not in several others. Successful subcloning was confirmed by restriction digestion, as the variant fragment possesses a new restriction site (*Pvu*I), as well as by sequencing. Several clones of these mutant constructs were used to transfect C300 and QT6 cells. The RT activity of the virus rescued from chicken cells rose rapidly, whereas a delay was evident in the time to peak viral activity in four different transfections of QT6 cells (Fig. 5). Rescued virus from both chicken and quail cells was also able to infect fresh chicken and quail cells with similar kinetics.

Analysis of receptor usage. To determine the receptor used by the variant virus, superinfection resistance assays were performed with QT6 and C300 cells infected with viruses with various host ranges. Cells were preinfected with viruses of various subgroups. After preinfection, these cells presumably express reduced levels of a subgroup-specific viral receptor on the plasma membrane and thus are less susceptible to superinfection with a virus which uses the same receptor. For preinfection, we used subgroup A (RAV-1) and B (td-PR-RSV-B) viruses on C/E cells, subgroup A and E (PR-E) viruses on QT6/BD cells, and a subgroup B/E recombinant virus, NTRE-4, on both cell types. NTRE-4 has been shown to recognize both the subgroup B receptor on chicken cells and the subgroup E receptor on quail cells (33). The preinfected cells were subsequently superinfected with a *lacZ* virus that had been packaged with a subgroup A or B/E virus or a mutant virus (S20). If the variant S20 virus used the same receptor as the virally infected cell, then there would be little infection, as indicated by a paucity of cells staining positive for β-galactosidase. If, however, a different receptor were used, there would be no receptor interference and thus more infection and staining.

The results of this experiment (Table 1) show that the mutant S20-*lacZ* virus pseudotype had a much lower titer on chicken cells that had been preinfected with td-PR-RSV-B or NTRE-4, suggesting that the chicken subgroup B receptor is

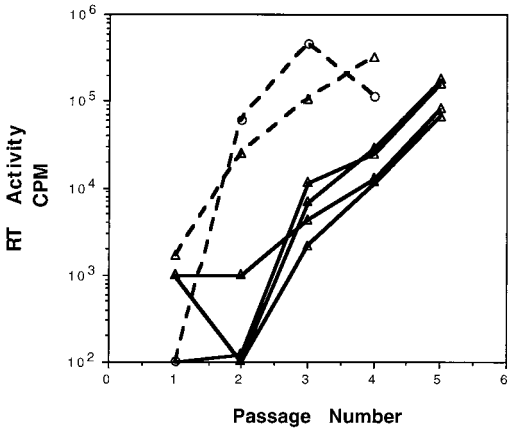

FIG. 5. Growth of the host range variant virus in chicken and quail cells. Reconstructed td-Pr-RSV-B DNA containing the 1.1-kb *Kpn*I to *Sal*I fragment from Pr-RSV-B (○) or S20 (△) was transfected into chicken (dashed lines) and quail (solid lines) cells. The RT activity of the rescued virus was monitored at subsequent passages. Four independent transfections of QT6 cells with the reconstructed mutant DNA are shown. QT6 cells transfected with wild-type Pr-RSV-B continued to show baseline RT activity at passage 3, after which passaging was discontinued.

TABLE 1. Receptor usage by the mutant virus

Preinfecting virus	No. of stained cells following superinfecting *lac* virus (cell type)[a]			
	S20 (C/E)	S20 (QT6/BD)	NTRE-4 (C/E)	RAV1 (C/E)
None	1,280	3,600	2,560	2,448
RAV-1	992	1,120	2,528	<5
td-Pr-RSV-B	16	ND	<5	1,920
Pr-RSV-E	ND	240	ND	ND
NTRE-4	<10	<5	<5	1,590

[a] C/E or QT6 cells were preinfected with the viruses shown and passaged three or four times prior to superinfection; the results reflect the number of stained cells per 60-mm plate 2 days after superinfection. ND, not done.

used by this mutant virus. Infection of QT6 cells by the mutant virus was blocked by preinfection with PR-E or NTRE-4, suggesting that the E receptor on quail cells is utilized. Thus, the extended receptor usage of this virus resembles that of NTRE-4.

DISCUSSION

Avian and murine retroviruses display considerable variability of receptor usage, despite strong conservation of most of the Env proteins (5, 6, 15, 24). The major host range determinants of avian retroviruses reside in the variable *hr*1 and *hr*2 regions in the middle third of SU (gp85) (Fig. 1). Previously, several recombinants, generated either in tissue culture or by recombinant DNA techniques, have shown that mixing of certain *hr*1 and *hr*2 regions from different subgroups can yield a virus that combines the receptor specificity of both parents. One such virus is NTRE-4, a recombinant between td-PR-RSV-B and the endogenous subgroup E virus RAV-0. This virus possesses 200 bases of the RAV-0 sequence, including *hr*2 but not *hr*1, within a td-PR-RSV-B framework; it recognizes both subgroup B and E receptors (15). These data have suggested a model in which *hr*1 and *hr*2 encode two separate segments of protein which together form the receptor binding site. Further, it is likely that only certain combinations of *hr*1 and *hr*2 can interact correctly with each other and/or the cell surface receptor to initiate infection, since many recombinants carrying novel combinations of variable regions fail to produce infectious virus following transfection (14).

We describe here a variant virus that grew to dominate the viral population under selective pressure for an expanded host range and which has dual specificity for the subgroup B and E receptors. Complete and reciprocal interference with NTRE-4 (Table 1) implies identical receptor usage by these two viruses, despite their different genetic structures. Consistent with this, both viruses were completely blocked by infection of chicken cells with a subgroup B virus. However, the mutant virus was significantly, but not completely, inhibited by preinfection of QT6 cells with Pr-E RSV, raising the possibility that it (and, by extension, NTRE-4) might use additional receptors present on QT6, but not chicken, cells. Further experiments are required to test this speculation.

The variant virus possesses two amino acid changes, both within the *hr*1 region. The first, at codon 155, substitutes a polar serine for the nonpolar leucine conserved among subgroup A, B, C, and E viruses. The second change substitutes nonpolar isoleucine for polar threonine at codon 156; it is of note that RAV-0 also possesses an Ile at codon 156, suggesting that the Ile at this position may be critical for subgroup E receptor use. In NTRE-4, which also recognizes the subgroup B and E receptors, the contribution of subgroup E specificity is encoded by *hr*2. The contrast between the two supports the notion that host range may be determined by the interaction of *hr*1 and *hr*2 with each other and/or with the cell receptor, in addition to a specific sequence. These findings indicate that altered receptor usage can be conferred by small changes in *env* and may also point to a key region for receptor interaction. Interestingly, it has previously been shown that in subgroup A avian sarcoma-leukosis virus, small, site-directed mutations in *hr*2 result in loss of subgroup A specificity whereas *hr*1 seems to tolerate small modifications without a change in specificity (35). Taken with our data, these observations may further support a role for *hr*1 and *hr*2 in conformational changes or envelope folding that may be critical to receptor binding.

A phenotypically variant virus with an extended host range was recognizable in the virus population at viral passage 29. However, when we cloned and sequenced the virus from QT6 cells infected with viral passages 28 and 32, only the wild-type virus was apparent. Failure to observe the mutant virus in the mixed population probably resulted from pseudotype formation of wild-type genomes by variant virus to allow infection of nonpermissive cells. We believe it unlikely that mutations in *env* other than the ones we describe here are responsible for the phenotypic host range alteration, since a cloned virus containing the two amino acid changes within an otherwise wild-type virus revealed the same phenotype as the passage 28 and 32 viruses upon transfection of QT6 cells. To eradicate the pseudotyping, as well as increase the selective pressure, we infected QT6lac914 cells with viral supernatant from viral passage 40 and serially passaged those cells, allowing only the variant to spread through the quail cell cultures. We suspect that the mutant virus, initially present at a low level, spread slowly through the QT6lac914 cells. By passage 9, there was a pronounced increase in RT activity as maximal levels of virus production were achieved. All of the clones analyzed after this passage possessed the two amino acids changes that were subsequently shown to be sufficient for the altered phenotype.

Interestingly, when mutant (S20) constructs were used to transfect chicken and quail cells, the rescued virus grew much more rapidly on chicken cells than on quail cells. This suggests greater fitness of the virus on chicken than on quail cells. Because the virus rescued from chicken and quail cells could subsequently infect both cell types with similar kinetics, it is unlikely that a mutation arose after rescue. It is likely, however, that continued passage of the variant virus on QT6 cells will improve its infectivity.

The extensive genetic diversity of retroviruses and the forces which act upon this variation are two of the important features that are responsible for the quasispecies nature of retroviral populations (11, 18). It seems likely that the ability of retroviruses to evolve rapidly in response to selective pressures imposed by the host (such as the development of genetic resistance) allowed retroviruses to acquire the ability to use different receptors. In our in vitro system, appropriately applied selective pressure did, in fact, lead to the appearance and growth of a variant virus with an altered host range. Thus, our data support the supposition that selective pressure imposed by polymorphic host cell receptors may have resulted in variable receptor usage by generally highly conserved envelope proteins. Analysis of the kinetics of appearance of the point mutations should reveal the strength of the selection; preliminary experiments using relatively insensitive methods indicate that the frequency of the mutations is quite low until shortly before the mutant virus becomes detectable. We are currently developing more sensitive techniques to address this issue.

The two mutations we observed were clearly sufficient to

confer the ability to use the quail subgroup E receptor on the subgroup B virus. The relatively long delay in the appearance of these mutations, combined with the reciprocal nature of the amino acid changes, suggests that each may be deleterious to virus replication when present independently. This hypothesis is being tested.

Recent work has suggested that similar residues on otherwise unrelated receptors are important for viral recognition. Specifically, an aromatic residue in the ALV-A receptor, CD4, and the ecotropic murine leukemia virus receptor appear to be critical for retrovirus-receptor interaction (1, 7, 27, 39). It is possible that the altered region of *hr*1 in our mutant virus contributes to a receptor recognition motif which recognizes a similar Env recognition region on the subgroup B and E receptors. Recently, the receptor for the subgroup B virus has been cloned and identified as a cell surface protein related to the Fas receptor and unrelated to the subgroup A receptor (8). Future study of wild-type subgroup B virus and our mutant virus in the context of this receptor should elucidate features of the Env-receptor interaction of these retroviruses.

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