Mutations in the *env* Gene of Friend Spleen Focus-Forming Virus Overcome *Fv-2^r*-Mediated Resistance to Friend Virus-Induced Erythroleukemia

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Although *Fv-2^r* homozygous mice are resistant to leukemias induced either by an erythropoietin-encoding virus or by wild-type Friend virus (FV) (M. E. Hoatlin, S. L. Kozak, F. Lilly, A. Chakraborti, C. A. Kozak, and D. Kabat, Proc. Natl. Acad. Sci. USA 87:9985–9989, 1990), they are susceptible to some variants of FV (R. A. Steeves, E. A. Mirand, A. Bulba, and P. J. Trudel, Int. J. Cancer 5:349–356, 1970; R. W. Geib, M. B. Seaward, M. L. Stevens, C.-L. Cho, and M. Majumdar, Virus Res. 14:161–174, 1989). To localize the virus gene involved in influencing the host range, we cloned and sequenced the *env* gene of the BB6 variant of FV (Steeves et al., Int. J. Cancer 5:349–356, 1970). In comparison with the wild-type *env* gene, the BB6 variant contains a 159-bp deletion that eliminates the membrane-proximal portion of the extracellular domain and 58 point mutations resulting in 13 amino acid changes. Substitution of the variant *env* gene for the wild-type *env* gene resulted in a recombinant virus that produced a Friend virus-like disease in *Fv-2^r* homozygotes. Our results identify the spleen focus-forming virus *env* gene as the viral gene involved in this virus-host interaction. Additionally, they suggest that the product of the *Fv-2^r* gene modifies the interaction between the spleen focus-forming virus envelope protein and the erythropoietin receptor.

Friend virus (FV) induces a rapid erythroleukemia in susceptible mice (14). FV is composed of at least two viruses: Friend murine leukemia virus (F-MuLV), a replication-competent virus, and Friend spleen-focus forming virus (SFFV), a replication-defective virus (4, 10, 45). F-MuLV causes a relatively rapid erythroleukemia in neonatal but not adult mice (34, 49). SFFV causes the characteristic acute erythroleukemia in both newborn and adult susceptible mice (50).

SFFV has a recombinant *env* gene composed of the 5' region from an endogenous dual-tropic virus and the 3' region from the ecotropic F-MuLV (1, 5, 9, 39, 40, 49, 54). During the evolution of the SFFV, a 585-bp deletion occurred in the *env* gene resulting in the fusion of gp70 and p15E. The product of the fusion, gp52, retained the transmembrane sequence of p15E. Additional changes believed to be essential for pathogenesis include a single-base insertion that shortens the reading frame by 33 amino acids and a 6-bp tandem repeat that adds two leucine residues (1, 2, 5, 9, 40, 54). The gp52 molecule is processed inefficiently from the rough endoplasmic reticulum to form a 65-kDa moiety that occurs on the cell surfaces (11, 42).

Recent evidence indicates that gp52 stimulates proliferation of an early erythroid progenitor cell, probably late

burst-forming unit-erythroid (BFU-E) or early colony-forming unit-erythroid (CFU-E), by binding to the erythropoietin receptor (Epo-R) (20, 30, 41, 55). The consequence is Epo-independent proliferation of the target cells. Although the mature form may be essential both for the induction of leukemia in vivo (28, 29, 32, 33, 42) and for erythroblast proliferation in vitro (5, 21), both forms of gp52 can apparently interact with Epo-R (6, 26).

Susceptibility to FV-induced leukemogenesis is influenced by several genes including *Fv-1*, *Fv-2*, *H-2*, and *Rfv-2* (48). *Fv-2* is unique among the genes controlling susceptibility because it down-regulates virus production and inhibits SFFV-induced foci formation and mitogenesis, yet it is only functional in erythroprogenitor cells from homozygous *Fv-2^r* mice (12, 31, 38). Suzuki and Axelrad (47) showed that the *Fv-2* gene is involved in controlling the development of hematopoietic cells. Later, Axelrad et al. (3) reported the purification of a negative regulatory protein from the bone marrow of *Fv-2^r* mice that down-regulates BFU-E DNA synthesis. They postulated that this down-regulation decreases the number of target cells available for FV infection.

Several variants of FV are host-range adapted to produce an FV-like disease in *Fv-2^r* mice: BB6 (46), BSB (46), RBV (17), and EYAB6 (18). The disease in *Fv-2^r* mice shares many characteristics of the classical FV disease in *Fv-2^{ss}* mice including splenomegaly, polycythemia, and transformation of erythroprogenitor cells (16, 17, 46). However, the latency period in *Fv-2^r* mice ranges from 1 to 6 months depending on the strain of virus and the strain of mouse used (36). Analysis of FV variants that have the ability to cause FV-induced disease in *Fv-2^r* mice provides one possible approach to understanding the interaction between the *Fv-2* gene and SFFV. Here we report the cloning and sequencing of the *env* gene of BB6 and demonstrate that the BB6 *env*

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gene is responsible for overcoming the resistance barrier of the Fv-2 gene.

MATERIALS AND METHODS

Mice. BALB/c (Fv-2^{ss}), B.C-H7^b/By (Fv-2^{ss}, congenic with C57BL/6), and C57BL/6 (Fv-2^r) mice were purchased from Jackson Laboratory, Bar Harbor, Maine. The strains of mice were bred in the animal facility of the Indiana University Terre Haute Center for Medical Education, Terre Haute. The animal facility conforms to all U.S. Department of Agriculture and National Institutes of Health regulations concerning animal care and welfare. All mice used in this study were at least 10 weeks old.

Virus. BB6 virus was obtained from Robert Eckner and Kristine Eckner (Boehringer Ingelheim, Ridgefield, Conn.) as a 20% spleen homogenate from C57BL/6 mice. Virus stocks were stored as 10 to 20% (wt/vol) spleen homogenate or as cell-free culture supernatant of virus-transformed cell lines. Spleens were removed from virus-infected mice, weighed, and homogenized in ice-cold phosphate-buffered saline (PBS). The homogenate was clarified at 10,000 × *g* for 10 min at 4°C. The supernatant was aliquoted and stored at -70°C. Cell-free tissue culture supernatant was harvested from cultures that were 80% confluent and stored at -70°C. Viruses were maintained by intravenous injection of virus in mice.

Hematocrit. Blood from virus-injected and normal mice was obtained from the retro-orbital sinus by using heparinized micro-hematocrit capillary tubes. The tubes were centrifuged for 3 min in a Triac centrifuge (Becton Dickinson and Co.). Packed cell volume was calculated by using a Critocap micro-hematocrit tube reader.

Cell lines. Cell lines from FV-infected spleens were established by the following method. Virus stocks were diluted with cold PBS and injected into mice intravenously through the lateral tail vein. Mice with enlarged spleens (2 to 3 g) were sacrificed, and the spleens were aseptically removed. Part of each spleen was used to make a single-cell suspension in Iscove's modified Dulbecco's medium supplemented with penicillin, streptomycin, 7 × 10⁻⁵ M 2-mercaptoethanol, and 30% fetal bovine serum. Within 3 to 4 days, cells were seen dividing. After the cell lines were established, the percentage of fetal bovine serum was decreased to 10% for maintenance. The remaining portion of each spleen was used to make a viral stock preparation. The IC-4 cell line was obtained from Robert Eckner and Kristine Eckner. This nonproducer cell line was made by infecting newborn rat kidney (NRK) cells with the BB6 virus. The BB6.B6 cell line was established by injecting BB6 virus in C57BL/6 mice. FVT-NB/A is an erythroleukemia cell line of BALB/c origin (13).

Induction of hemoglobin synthesis. FV-induced cell lines were cultured in Iscove's modified Dulbecco's medium with or without 1 to 1.8% dimethyl sulfoxide (DMSO) at an initial concentration of 10⁵ cells per ml. After 5 days, 0.25 ml of the cells was transferred into separate wells in a 24-well plate. Fresh benzidine reagent (0.1% final concentration) was added to the cells. At least 100 cells were scored for blue coloration by using a hemocytometer (15).

Analysis of envelope protein. Cells in the logarithmic growth phase were incubated with [³H]leucine at 100 μCi/ml for 1.5 to 2 h (16). After three washes in cold PBS, the cells were lysed in extraction buffer containing 0.01 M sodium phosphate buffer (pH 7.6), 0.1 M sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dode-

cyl sulfate (SDS). The cell extracts were centrifuged at 10,000 × *g* for 10 min at 4°C, cleared with normal goat serum, incubated with a 10% preparation of *Staphylococcus aureus* Cowan strain I (Staph A) for 1 to 2 h at 4°C, and clarified by centrifugation at 10,000 × *g* for 5 min. After incubation with goat anti-Rauscher gp70 antiserum, immunoprecipitation was facilitated by incubation with Staph A for 2 h at 4°C. Immunoprecipitates were pelleted, washed three times with extraction buffer, resuspended in 2× sample buffer (1× sample buffer contained 0.05 M Tris [pH 6.8], 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue), and boiled for 4 min. Samples were applied to 7 or 10% SDS-polyacrylamide gels (27). The gels were fixed, dried, and autoradiographed at -70°C.

Amplification of envelope gene. Polymerase chain reaction (PCR) was utilized to amplify the viral *env* gene. Hirt DNA (22) prepared from NIH 3T3 cells infected with BB6 virus was used as a template in a 100-μl reaction containing PCR buffer (10 mM Tris [pH 8.3], 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.1% gelatin), deoxyribonucleotides (200 μM each deoxyribonucleotide), primers (0.2 to 1.0 μM) (5'-CGTGGATCCACGCCGCTCAGT-3' located immediately upstream of the 5' end of the *env* gene and 5'-CTGAGGAGACCCTCCCAAGG-3' located in the long terminal repeat), and 2.5 U of AmpliTaq DNA polymerase (United States Biochemical, Cleveland, Ohio). Each PCR cycle profile consisted of 1.25 min at 94°C (melting step), 2.5 min at 50°C (annealing step), and 3.0 min at 72°C (polymerizing step). The cycle was repeated 30 to 40 times.

The PCR-amplified DNA was analyzed by agarose gel electrophoresis and then by Southern hybridization (44). Hybridization was performed with the 18-mer oligonucleotide probe (5'-ATTTGGACCCTGTATTCT) according to the procedure of Moreau-Gachelin et al. (37).

Cloning of BB6 envelope gene. Amplified BB6 *env* gene fragments were extracted from agarose gel and digested with restriction enzymes *Bam*HI and *Kpn*I and ligated to similarly digested plasmid pT7/T3 alpha 18 (Bethesda Research Laboratories). Transformation was performed with the ligation mixture by the procedure of Hanahan (19). Plasmid DNA was prepared from positive clones by the rapid boiling method (24). For Southern hybridization analysis, recombinant clones were digested with restriction endonucleases *Bam*HI and *Kpn*I and analyzed by agarose gel electrophoresis, and hybridization was performed as described above. The plasmid carrying the cloned *env* genes of BB6 was designated pEBB6.

Sequencing. DNA sequencing was done by the dideoxy-chain termination method of Sanger et al. (43), using Sequenase version 2.0 (United States Biochemical), according to the procedure recommended by the manufacturer. Direct sequencing of double-stranded plasmid DNA was performed (52). The nucleotide sequence data were pooled and manipulated by using the Microgenie program from Beckman (Palo Alto, Calif.).

Subcloning of envelope gene of BB6. The plasmid pSFF used for subcloning had a modified SFFV DNA insert (23). The *env* gene from pSFF was replaced with the *env* gene of pEBB6, thus engineering a complete recombinant BB6 viral DNA (see Fig. 4). This was done by digesting plasmid pEBB6 with the restriction enzymes *Bam*HI and *Kpn*I. The digestion released the *env* gene, which was gel purified and ligated to similarly digested pSFF. The recombinant thus constructed was called pSBB6.

Transfection of pSBB6. Transfection was performed with Lipofectin (Bethesda Research Laboratories) and by the

TABLE 1. Hemoglobin production in cell lines

Cell line	Percentage of benzidine-positive cells with the following amt of DMSO:					
	0%	1%	1.2%	1.4%	1.6%	1.8%
FVT-NB/A ^a	<1	ND ^b	ND	40	ND	ND
BB6.B6	<1	8	10	10	9	7

^a FVT-NB/A is an FV-induced tumor cell line of BALB/c origin (13).

^b ND, not determined.

procedures recommended by the manufacturer. The cells used for transfection were NIH 3T3 cells infected with the helper virus F-MuLV. Subconfluent cells were washed three times with Dulbecco's modified Eagle's medium without serum, and 3 ml of the same medium was added to the cells. Lipofectin and the plasmid DNA (pSBB6 and pSV^{neo}) were mixed and incubated at room temperature for 15 min. The mixture was added to the cells, and the cells were allowed to grow overnight. G418-resistant colonies of cells were transferred into 24-well plates and screened for the presence of viral envelope proteins. Cell-free supernatants from positive clones were injected into mice to check for virus production.

Nucleotide sequence accession number. The sequences described in this report have been deposited in GenBank under accession number M90673.

RESULTS

Induction of erythroid cell lines. The ability of BB6 virus to induce an erythroleukemia was tested by injecting BB6 virus into adult C57BL/6 mice, harvesting the enlarged spleens, and placing the splenocytes in Iscove's modified Dulbecco's medium (16). The resulting cell line was termed BB6.B6. The cell lines established from mice infected with wild-type FV are erythroid and can often be induced to differentiate and produce hemoglobin by culturing them in presence of DMSO (15). To demonstrate that the BB6.B6 cell line was erythroid, it was cultured in 1 to 1.8% DMSO and stained with benzidine to detect hemoglobin (15). In the presence of 1.2 to 1.4% DMSO, 10% of cells had detectable levels of hemoglobin (Table 1).

Analysis of envelope proteins of BB6. The envelope proteins produced in the BB6-induced cell line (BB6.B6) were compared with the envelope proteins produced in IC-4 (an NRK cell line infected with BB6 virus) and FVT-NB/A (an erythroleukemia cell line induced with wild-type FV [13]) by immunoprecipitation of the proteins with goat anti-Rauscher gp70. As seen in Fig. 1, both the BB6.B6 and the IC-4 cell lines produced an SFFV-related envelope protein with an apparent M_r of 42,000. This is in contrast to the 52-kDa SFFV envelope protein produced in the FVT-NB/A cell line. The higher-molecular-weight protein bands correspond to envelope proteins of the F-MuLV and Friend mink cell focus-forming helper viruses.

Amplification and cloning of envelope gene. To further characterize the SFFV *env* gene, we amplified the *env* gene and long terminal repeat of the BB6 virus by PCR from the Hirt DNA of BB6-infected cells. The primers used in the PCR assay amplified only SFFV-related sequences since they hybridized to a 5' mink cell focus-forming virus sequence and a 3' ecotropic sequence. The amplification product was demonstrated to be SFFV related by agarose gel electrophoresis and subsequent Southern blot analysis with an SFFV-specific probe (37). As predicted from the

FIG. 1. Comparison of the BB6 SFFV envelope protein with the wild-type SFFV envelope protein. The SFFV envelope proteins of the BB6 variant and the wild-type FV were compared by immunoprecipitation of biosynthetically labeled cell lysates from the following cell lines: FVT-NB/A (a BALB/c [*Fv-2^{ts}*] erythroleukemia cell line induced with wild-type FV); IC-4 (NRK cells infected with BB6 virus); and BB6.1 (a C57BL/6 [*Fv-2^{ts}*] erythroleukemia cell line induced with BB6 virus). Cell lysates were cleared with normal goat serum and Staph A, and this was followed by specific precipitation with goat anti-Rauscher gp70. The precipitates were analyzed by electrophoresis on a 7.5% polyacrylamide gel. Numbers on left show molecular weight ($\times 10^3$).

protein data, the amplified BB6 *env* gene plus the long terminal repeat (1.6 kb) was smaller in size than the wild-type SFFV (1.8 kb) *env* gene plus the long terminal repeat. The amplified *env* gene was cloned between the *Kpn*I and *Bam*HI sites of pT7/T3 alpha 18.

Sequencing and amino acid analysis. The BB6 *env* gene was sequenced in both directions with double-stranded DNA from the plasmid pEBB6. Each region was sequenced twice to confirm the results. The sequences were read and processed by using a GelMate 1000 Sonic Digitizing System (Beckman) and analyzed by using a MicroGenie Sequence Software program (Beckman). The BB6 *env* gene is 1,068 bp long (Fig. 2). This is 159 bp shorter than the wild-type *env* gene. The BB6 *env* gene retained the following characteristics of the wild-type SFFV *env* gene: a 585-bp deletion at the 3' end; a single-base insertion that prematurely terminates the coding sequence; and a 6-bp duplication that leads to the addition of two leucine residues. The distinguishing features of the BB6 *env* gene include a deletion of 159 bp at the 3' end that increases the original deletion of 585 bp to 744 bp, and 58 point mutations.

—Leader peptide—
 ATG GAA GGT CCA GCA TTC TCA AAA CCC CTT AAA GAT AAG ATT AAC CCG TGG GGC CCC CTA 60
 Met Glu Gly Pro Ala Phe Ser Lys Pro Leu Lys Asp Lys Ile Asn Pro Trp Gly Pro Leu
 —NH₂terminus, gp45—
 ATA GTC CTG GGG ATC TTA ATA AGG GCA GGA GTA TCA GTA CAA CGT GAC AGC CCT CAT CAG 120
 Ile Val Leu Gly Ile Leu Ile Arg Ala Gly Val Ser Val Gln Arg Asp Ser Pro His Gln
 GTC TTC AAT GTT ACT TGG AGA GTT ACC AAC TTA ATG ACA GGA CAA ACA GCT AAT GCT ACC 180
 Val Phe Asn Val Thr Trp Arg Val Thr Asn Leu Met Thr Gly Gln Thr Ala Asn Ala Thr
 TCC CTC CTG GGG ACA ATG ACC GAT GCC TTT CCT AAA CTG TAC TTT GAC TTG TGC GAT TTG 240
 Ser Leu Leu Gly Thr Met Thr Asp Ala Phe Pro Lys Leu Tyr Phe Asp Leu Cys Asp Leu
 ATA GGG AAC GAC TGG GAT GAG ACT AGA CTC GGG TGT CGC ACT CCC GGA GAA GGA AAA AGG 300
 Ile Gly Asn Asp Trp Asp Glu Thr Arg Leu Gly Cys Arg Thr Pro Gly Glu Gly Lys Arg
 GCA AGA ACA TTT GAC TTG TAT GTT TGC CCC GGG CAT ACT GTA CCA ACA GGG TGT GGA GGG 360
 Ala Arg Thr Phe Asp Leu Tyr Val Cys Pro Gly His Thr Val Pro Thr Gly Cys Gly Gly
 CCG AGA GAG GGC TAC TGT GGC AAA TGG GGC TGT GAG ACC ACT GGA CAG GCA TAC TGG AAG 420
 Pro Arg Glu Gly Tyr Cys Gly Lys Trp Gly Cys Glu Thr Thr Gly Gln Ala Tyr Trp Lys
 CCA TCA TCA TCA TGG GAC CTA ATT TCC CTT AAG CGA GGA AAC ACT CCT AAG GAT CGG GGC 480
 Pro Ser Ser Ser Trp Asp Leu Ile Ser Leu Lys Arg Gly Asn Thr Pro Lys Asp Arg Gly
 CCC TGT TAT GAT TCC TCG GTC TCC AGT GGC GTC CAG GGC GCC ACA CCG GGG GGT CGA TGC 540
 Pro Cys Tyr Asp Ser Ser Val Ser Ser Gly Val Gln Gly Ala Thr Pro Gly Gly Arg Cys
 AAC CCC CTG GTC TTA AAA TTC ACT GAC GCG GGT AAA AAG GCC AGC TGG GAT GCC CCC AAA 600
 Asn Pro Leu Val Leu Lys Phe Thr Asp Ala Gly Lys Lys Ala Ser Trp Asp Ala Pro Lys
 GTA TGG GGA CTA AGA CTG TAC CGA TCC ACA GGG ACC GAC CCG GTG ACC CGG TTC TCT TTG 660
 Val Trp Gly Leu Arg Leu Tyr Arg Ser Thr Gly Thr Asp Pro Val Thr Arg Phe Ser Leu
 ACC CGC CAG GTC CTC AAT ATA GGG CCC CGC GTC CCC ATT GGG CCT AAT CCC GTG ATC TCT 720
 Thr Arg Gln Val Leu Asn Ile Gly Pro Arg Val Pro Ile Gly Pro Asn Pro Val Ile Ser
 GAC CAG TTG CCC CCC TCC CGA CCC GCG CAG ATC ATG CTC CCC AGG CCT CCT CAG CCT CCT 780
 Asp Gln Leu Pro Pro Ser Arg Pro Ala Gln Ile Met Leu Pro Arg Pro Pro Gln Pro Pro
 CCT CCA GGC ACA GCC TCT ATA GTC CCT GAA ACT GCC CCA CCT TCT CAA CAA CCT GGG ACG 840
 Pro Pro Gly Thr Ala Ser Ile Val Pro Glu Thr Ala Pro Pro Ser Gln Gln Pro Gly Thr
 AGA GAC AGG CTG CTA AAC CTG GTA AAT AAA GCC TAC CAA GCT CTC AAC CTC ACC AGT CCT 900
 Arg Asp Arg Leu Leu Asn Leu Val Asn Lys Ala Tyr Gln Ala Leu Asn Leu Thr Ser Pro
 —Recombination Site—
 GAC AAA ACC CAA GAG TGC TGG TTA TGC CTA GTG TCT AGA CCC CCC TAT TAC GAG GGG GTT 960
 Asp Lys Thr Gln Glu Cys Trp Leu Cys Leu Val Ser Arg Pro Pro Tyr Tyr Glu Gly Val
 —(gp70)—(p15E)— HYDROPHOBIC REGION
 GCG GTC CTA GGC ACT AAT TCT AAT CAT ACC ACG TTG ATA TCC ACC ATC ATG GGG CTT CTC 1020
 Ala Val Leu Gly Thr Asn Ser Asn His Thr Thr Leu Ile Ser Thr Ile Met Gly Leu Leu
 ATT ATA CTC CTA CTC CTA CTA ATT CTG CTT TTG TGG ACC CTG CAT TCT TAA 1071
 Ile Ile Leu Leu Leu Leu Leu Ile Leu Leu Leu Trp Thr Leu His Ser End

FIG. 2. Nucleotide sequence of the BB6 *env* gene. The BB6 *env* gene was sequenced from the plasmid pEBB6. The DNA was sequenced twice in both directions. The deduced DNA sequence and the corresponding amino acid sequence are shown. The site of the 159-bp deletion begins at the junction between gp50 and p15E. The potential glycosylation sites are indicated by shadowing. The hydrophobic region is indicated by underlined bold lettering.

The nucleotide sequence of the BB6 *env* gene was converted to an amino acid sequence by using the MicroGenie translation program. The predicted amino acid sequence of the BB6 envelope protein was compared with the predicted sequence of three known wild-type SFFV envelope proteins (Fig. 3). In comparison with the wild-type envelope protein of SFFV, which is 409 amino acids long, the BB6 envelope protein is 356 amino acids long. The 58 point mutations in the BB6 *env* gene resulted in 13 amino acid changes in the BB6 protein.

Biological activity of cloned *env* gene. The BB6 *env* gene was cloned into pSFF by the strategy shown in Fig. 4 and termed pSBB6. pSBB6 was cotransfected with pSV^{neo} into NIH-F-MuLV cells to rescue the recombinant virus. After selection for resistance to G418, the colonies were screened for the presence of BB6 envelope protein by immunoprecipitation with goat anti-Rauscher gp70. Several clones were expanded and used in subsequent studies.

To test the hypothesis that the BB6 *env* gene is responsible

for the disease seen in resistant mice, the rescued virus was harvested from the cell-free supernatant and injected into mice by two different protocols. In the first protocol, 0.5 ml of supernatant was injected intravenously into three strains of mice, C57BL/6, B.C-H7^b/By, and BALB/c. Nine days later, the experiment was terminated. Hematocrits, spleen weights, and foci counts were determined (Table 2). In the second protocol, the mice were injected twice with a two-fold-concentrated supernatant to induce long-term FV disease (Table 3). Nine days postinfection, a mild splenomegaly was observed in BALB/c mice (0.15 g versus 0.10 g in normal mice) and C57BL/6 mice (0.16 g versus 0.08 g in normal mice) (Table 3). Foci were observed in both strains of mice (Table 2). Several animals of both strains were monitored by palpation for splenomegaly for more than 6 months. Massive splenomegaly was observed in BALB/c mice by 44 days. Polycythemia developed concurrently with splenomegaly. The disease in C57BL/6 mice was more protracted and somewhat variable. In some experiments, only two of

FIG. 3. Comparison of the predicted amino acid sequence of the BB6 SFFV envelope protein with that of three wild-type SFFV envelope proteins. The standard one-letter abbreviations for amino acids are used. The sequences for the wild-type SFFV are based on the following: SFFV52, Wolff et al. (54); SFFV55, Amanuma et al. (1); and SFFVan, Wolff et al. (53). Dots indicate identity with the SFFV52 sequence. Stippled areas indicate deletions compared with the SFFV52 sequence. Solid arrowheads indicate a BB6-specific amino acid.

six mice maintained for long-term examination developed disease. However, in mice developing splenomegaly, the spleen weights could exceed 2 g.

DISCUSSION

Comparison of the structural features of the BB6 envelope protein with those of the wild-type SFFV envelope protein. Comparison of biologically important regions of the wild-

type virus with similar regions of host-range variants is one mechanism to identify regions of the virus genome that influence the host range. The 5' region of the SFFV *env* gene encodes a highly conserved domain that appears to be essential for pathogenicity (25, 28, 29). This conserved domain may be involved in binding to the Epo-R (25). Comparison of the BB6 envelope protein with the wild-type envelope protein indicates that BB6 has several unique amino acid changes within the conserved domain. The

Recent studies indicate that the 3' region of the SFFV *env* gene has several features that are critical for pathogenesis. These include a 6-bp tandem repeat; a single base pair insertion; and a 585-bp deletion that fuses the gp70 and p15E molecules. BB6 virus retained the 6-bp repeat and the single base pair insertion but increased the 585-bp deletion to 744 bp. Because three other variants, BSB (8, 46), RBV (17, 35), and EYAB6 (18), have deletions similar to those seen with BB6, we conclude that deletions in this area of the SFFV *env*

Mechanism of disease induction by the BB6 strain of FV in *Fv-2^{ss}* mice. In the *Fv-2^{ss}* environment, normal erythroid proliferation occurs by the binding of Epo to Epo-R. Infection of erythropoietic cells with SFFV leads to the synthesis of gp52 and its interaction with the Epo-R, either on the surface or intracellularly (30, 41, 55). This interaction mimics Epo-Epo-R binding and causes Epo-independent proliferation. The disease induced by BB6 in *Fv-2^{ss}* mice maintains many of the features associated with the wild-type

TABLE 2. Changes in erythrocyte volume, spleen weight, and foci 9 days postinfection with the cloned BB6 variant of FV^a

Mouse strain	Dilution	Hematocrit ($\pm$ SD)	Spleen wt ($\pm$ SD) (g)	No. of foci ^b
Expt 1				
B.C-H7 ^b /By	No virus	51.3 ($\pm$ 1.2)	0.085 ($\pm$ 0.005)	ND
	1:5	52.3 ($\pm$ 0.6)	0.140 ($\pm$ 0.008)	Confluent
	1:10	50.3 ($\pm$ 0.6)	0.126 ($\pm$ 0.019)	Confluent
	1:100	49.7 ($\pm$ 0.6)	0.119 ($\pm$ 0.025)	Confluent
	1:1,000	50.0 ($\pm$ 1.0)	0.126 ($\pm$ 0.033)	TMTC
C57BL/6	No virus	52.0 ($\pm$ 1.0)	0.073 ($\pm$ 0.0004)	ND
	1:5	50.3 ($\pm$ 1.2)	0.111 ($\pm$ 0.029)	Confluent
	1:10	47.0 ($\pm$ 1.0)	0.136 ($\pm$ 0.057)	Confluent
	1:100	50.3 ($\pm$ 1.5)	0.119 ($\pm$ 0.011)	Confluent
	1:1,000	50.0 ($\pm$ 1.4)	0.098 ($\pm$ 0.014)	TMTC
Expt 2				
BALB/c	No virus	47.3 ($\pm$ 0.5)	0.123 ($\pm$ 0.009)	ND
	Neat	47.0 ($\pm$ 0.0)	0.173 ($\pm$ 0.002)	Confluent
	1:10	43.5 ($\pm$ 3.5)	0.314 ($\pm$ 0.172)	Confluent
	1:100	48.0 ($\pm$ 1.4)	0.133 ($\pm$ 0.003)	Confluent
C57BL/6	No virus	52.0 ($\pm$ 1.0)	0.073 ($\pm$ 0.0004)	ND
	Neat	51.5 ($\pm$ 0.7)	0.182 ($\pm$ 0.029)	Confluent
	1:10	44.0 ($\pm$ 2.8)	0.143 ($\pm$ 0.001)	Confluent
	1:100	45.5 ($\pm$ 2.1)	0.132 ($\pm$ 0.025)	Confluent

^a Cell culture supernatants were harvested from F-MuLV-producing 3T3 cells transfected with the plasmid pSBB6 6 h after being given fresh medium (3 ml per confluent 100-mm plate). The supernatants were concentrated to 0.5 volume and injected intravenously (0.5 ml) into adult mice ($n = 3$ in each experiment).

^b ND, not done; Confluent, spleens confluent with foci; TMTC, discrete foci, but too many to count to obtain an accurate number.

FV disease including hepatosplenomegaly and transformation of erythropoietic cells. Analysis of the predicted amino acid sequence indicates that the BB6 *env* gene retained many of the features believed to be critical for pathogenesis. Therefore, it is likely that the BB6 virus induces disease in *Fv-2^{ss}* mice by the same mechanism used by the wild-type SFFV. Consistent with this expectation is the observation that the BB6 *env* gene confers factor independence to DA3/EpoR and BAF3/EpoR cells containing the Epo-R gene (26). On the other hand, the disease in C57BL/6 mice is variable and has a more protracted latency period. The extended latency period in C57BL/6 mice may be due to

a gene other than the *Fv-2* gene (17). Likely candidates include *H-2D^b*, *H-2I^b*, *Rfv-1*, or *Rfv-3* (7). This is currently being examined.

How does the *Fv-2^{ss}* gene mediate susceptibility to SFFV? Control of erythropoiesis in the *Fv-2^{ss}* environment appears to be different from control in the *Fv-2⁺* environment since both the wild-type F-SFFV and Epo virus (a retrovirus containing a functional *Epo* gene) are unable to induce disease in *Fv-2^{ss}* animals (23). Supporting this hypothesis is the observation that the BFU-E in the *Fv-2^{ss}* microenvironment do not cycle at the same rate as the BFU-E in the *Fv-2^{ss}* microenvironment (3). Several possibilities can be posited to explain the *Fv-2^{ss}* effect: the *Fv-2* gene may encode a second subunit of Epo-R that interacts with a conserved region of gp52 (the *Fv-2⁺* allele would then bind

FIG. 4. Subcloning strategy of the BB6 *env* gene into pSFF. The pSFF plasmid was digested with *Bam*HI (B) and partially digested with *Kpn*I (K). The fragment was purified by gel electrophoresis. The BB6 *env* gene was released from pEBB6 by digestion with *Bam*HI and *Kpn*I. The fragment was purified by gel electrophoresis and ligated to the linearized pSFF fragment. The recombinant was designated pSBB6. LTR, long terminal repeat.

TABLE 3. Long-term pathological changes induced with the cloned BB6 variant of FV^a

Mouse strain	Time after infection (days)	Hematocrit	Spleen wt (g)
BALB/c	9	49, 49, 48	0.15, 0.14, 0.16
	44	52, 63	1.90, 2.50
	53	72	2.74
	64	Died, 80	2.50, 4.80
C57BL/6	9	46, 44, 44	0.14, 0.21, 0.14
	144	81	1.61
	193	83	2.39

^a Mice were injected intravenously with BB6c13 cell culture supernatant (0.5 ml) on days 0 and 3. Eight mice were injected for each group. Three BALB/c and C57BL/6 mice were sacrificed on day 9. The remainder were terminated when splenomegaly was easily identifiable by palpation. Three C57BL/6 mice showed no signs of disease when the experiment was terminated at 193 days.

less effectively with gp52 and Epo); the *Fv-2^r* gene may encode a product that down-regulates the expression of the Epo-R; or the *Fv-2^r* gene may encode a protein that influences a step in proliferation occurring after Epo binds to its receptor.

It is unlikely that *Fv-2^r*-mediated resistance results exclusively from an Epo-R polymorphism that influences interaction with gp52. The reasons are as follows. Since the interaction between gp52 and Epo-R is believed to involve either the transmembrane or extracellular domain of the Epo-R (25, 55), it could be argued that changes within the conserved domain of the BB6 variant allow the variant to overcome *Fv-2^r*-mediated resistance by enhancing the gp52-Epo-R interaction. However, the analysis of other *Fv-2^r*-adapted variants negates this argument, since only two of four SFFV variants have significant changes within the conserved domain (8, 18, 35). Second, *Fv-2^{ss}* erythroleukemia cells transplanted into *Fv-2^r* mice require 3 to 6 months to produce tumors. This is in contrast to the rapid growth of tumors in the *Fv-2^{ss}* congenic mice (36). The growth pattern is indicative of a soluble growth-regulating factor in the *Fv-2^r* environment (possibly produced by T cells [51]). Interestingly, the *Fv-2^{ss}* erythroleukemia cells that do eventually adapt to produce tumors in resistant mice retain their wild-type *env* gene sequence.

Neither control of Epo-R expression nor control of signal transduction is inconsistent with experimental observations concerning the mechanism of the *Fv-2^r* gene. Analysis of the predicted amino acid sequence of the adapted variants indicates that each has a deletion near the transmembrane domain. The deletions in this area may influence either interaction between the receptor and gp52 or signal transduction. The observations that all the *Fv-2^r*-adapted variants have similar deletions and that the wild-type SFFV envelope protein interacts with the Epo-R (30, 41, 55) strongly imply that the *Fv-2^r* gene product may modify signal transduction originating at the Epo-R.

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