JOURNAL OF VIROLOGY, Feb. 1984, p. 394–402
0022-538X/84/020394-09$02.00/0

Vol. 49, No. 2

# Reduced Leukemogenicity Caused by Mutations in the Membrane Glycoprotein Gene of Rauscher Spleen Focus-Forming Virus

CURTIS A. MACHIDA, RICHARD K. BESTWICK, AND DAVID KABAT*

*Department of Biochemistry, School of Medicine, Oregon Health Sciences University, Portland, Oregon 97201*

Received 15 July 1983/Accepted 24 October 1983

We isolated and characterized two spontaneous, weakly leukemogenic mutants of Rauscher spleen focus-forming virus (R-SFFV) that contain mutations in nonoverlapping regions of the membrane envelope (*env*) glycoprotein gene. As reported previously (M. Ruta and D. Kabat, J. Virol. **35**:844–853, 1980), the replication-defective R-SFFV encodes a membrane glycoprotein with an apparent $M_r$ of 54,000 (gp54) which is structurally and immunologically related to the membrane envelope glycoproteins of dual-tropic murine leukemia viruses. Mutant R-SFFV clones 3-25 and 4-3 encode abnormally sized gp54-related glycoproteins with apparent $M_r$s of 52,000 (gp52) and 45,000 (gp45), respectively. Northern and Southern blot analyses of the mutant R-SFFV nucleic acids indicated that an insertion has occurred in the 3-25 *env* gene and that a deletion has occurred in the 4-3 *env* gene. Furthermore, restriction endonuclease analyses and comparisons of the fragmentation patterns of the wild-type and mutant glycoproteins generated by partial proteolysis with *Staphylococcus aureus* V8 protease indicated that the mutations affect nonoverlapping domains of the envelope glycoprotein (amino-terminal fragment affected in 3-25 glycoprotein and carboxyl-terminal fragment affected in 4-3 glycoprotein). Glycosylation inhibition studies indicated that the reduced size of gp52 is caused at least partly by loss of an asparagine-linked oligosaccharide. In addition, these mutant viruses have dramatically reduced leukemogenicities compared with wild-type R-SFFV. We conclude that the gp54 structural gene is required for initiation or amplification of the splenic erythroblast hyperplasia which characterizes the preleukemic phase of Rauscher disease.

The Rauscher and Friend erythroleukemia viruses are independently isolated complexes consisting of two viral components. One component is a replication-defective, highly pathogenic spleen focus-forming virus (SFFV) which as a pseudotype causes rapid formation of erythroid colonies in the spleens of susceptible mice and in cultures of bone marrow cells (3, 5, 25). The second component is a replication-component murine leukemia virus (MuLV) which by itself causes lymphatic leukemia in adult mice after a relatively long latency period (16, 22, 42). Infection of susceptible mice with the viral complexes causes an initial hyperplasia of erythroid cells, followed 3 to 8 weeks later by the emergence and dissemination of increasingly malignant erythroleukemia cells (35, 57). Consequently, the diseases induced by these viruses are similar to progressive natural cancers, which evolve from premalignant to malignant stages (23).

There is substantial evidence that the SFFVs and replication-competent dual-tropic MuLVs are closely related (8, 10, 20, 54), and it has been proposed that these viruses may be members of a single virus class which can cause diverse progressive hematopoietic neoplasms by common mechanisms (8, 10, 13, 18, 20, 54). For example, the Rauscher and Friend SFFVs (R- and F-SFFVs) encode membrane glycoproteins with apparent $M_r$s of 54,000 to 55,000 (gp54 and gp55 for R- and F-SFFV, respectively) which are structurally and immunologically related to the envelope glycoproteins encoded by dual-tropic murine leukemia viruses (17, 45). Although the *gag* and *pol* genes of F-SFFV contain a small substitution or partial deletion (10, 20), respectively, these same genes in F-SFFV appear to be structurally and functionally indistinguishable from those of dual-tropic MuLVs (8).

* Corresponding author.

Several lines of evidence have suggested that the gp54 and gp55 structural genes of SFFVs might perform a leukemogenic function. First, as indicated above, the independently isolated but pathogenically related R- and F-SFFVs contain similar *env* genes but widely divergent *gag* and *pol* genes (8, 48). Second, as mentioned previously, the SFFVs and dual-tropic MuLVs appear to be structurally related members of a single virus class (8, 10, 20). Moreover, the gp70 molecules encoded by the *env* genes of dual-tropic MuLVs have been indirectly implicated in leukemogenesis (10, 11, 14, 24, 30, 36, 55, 59). Third, transfection studies have been done with a molecularly cloned subgenomic fragment of F-SFFV DNA which contains the gp55 structural gene (31, 33). These studies have indicated that the subgenomic DNA fragment can recombine intracellularly in the presence of MuLV to form a pathogenically active "recovered" SFFV (31) and that in vitro mutagenesis of this genetic region by a deletion can eliminate its activity in the recovery assay (33). Fourth, spontaneous, transmissible F-SFFV mutants with abnormalities in their gp55 structural genes have recently been isolated (46). These mutants have dramatically reduced leukemogenicities compared with wild-type F-SFFV. We now report the first isolation and characterization of R-SFFV mutants; these mutants contain abnormalities in nonoverlapping regions of their gp54 structural genes and are only weakly leukemogenic.

## MATERIALS AND METHODS

**Cells, viruses, and origin of spontaneous R-SFFV mutants.** All cell lines were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal calf serum and antibiotics. The R-SFFV-infected NIH-3T3 nonproducer cell line, RV NIH clone 6, has been previously described (48). MuLVs were routinely titrated by using the

S+L− method (7). Virus from the RV NIH clone 6 nonproducer cell line was rescued by superinfection with a previously described clone of Rauscher MuLV (R-MuLV) (49) and was passaged through mice three times to amplify the R-SFFV titer. NIH-3T3 fibroblasts were infected with this passaged Rauscher virus preparation, and virus released from this infected cell line was used for isolation of viral mutants. Isolation of the spontaneous R-SFFV mutants (clones 3-25 and 4-3) was accomplished by limiting dilution cloning into NIH-3T3 fibroblasts and by screening the resulting fibroblast colonies for gp54-related glycoproteins as previously described (47, 51). In the process of this mutant screening, numerous wild-type R-SFFVs with electrophoretically normal gp54s were also isolated. One of the latter (clone 3-32) was often used as a wild-type R-SFFV standard. Consistent with previous descriptions (48), the pathogenic and biochemical properties of this wild-type R-SFFV were indistinguishable from those of other R-SFFVs that were clonally isolated throughout these studies.

**Labeling and extraction of cells, antisera, immunoprecipitation procedures, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** Methods for metabolic labeling of cells with L-[$^{35}$S]methionine or D-[$^{3}$H]glucosamine (38, 48), lactoperoxidase-catalyzed surface iodination with [$^{125}$I]iodine (27), inhibition of glycosylation with tunicamycin (34), immunoprecipitation of proteins from labeled cell extracts with antiserum (48), electrophoresis of proteins in the presence of sodium dodecyl sulfate (28, 48), and the fluorographic detection of radioactive protein components on the dried gels (9, 48) have been described previously.

The goat antisera to R-MuLV gp70 and to p30 and a rat antiserum which reacts with gp54 and with the gp70s of dual-tropic MCF247 and HIX MuLVs but not with the glycoproteins of ecotropic MuLVs have also been previously described (19, 27, 45).

**Protein fragmentation methods.** Partial proteolysis of R-SFFV glycoproteins with *Staphylococcus aureus* V8 enzyme (Miles Laboratories) were conducted essentially as described by Cleveland et al. (12) with minor modifications. Elution of glycoproteins from gel sections was performed by incubating the gels in water at 37°C for 18 h. The eluted glycoproteins were then concentrated in vacuo and digested with various concentrations of enzyme at 37°C for 30 min. The digestions were terminated by the addition of 2-mercaptoethanol, and the proteolytic fragments were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Nucleic acid analyses.** Methods for isolation of unintegrated proviral DNA from virus-infected cells (26), extraction of cytoplasmic RNA from cell lines (50), purification of polyadenylic acid-containing RNA by oligodeoxythymidylic acid-cellulose chromatography (4), electrophoresis of nucleic acids through agarose or methyl mercuric hydroxide gels (6, 8), blot transfer of nucleic acids from gels to diazophenylthioether paper (1, 52, 56), and preparation and use of hybridization probes (8, 39, 43) have all been previously described. For detection of SFFV-specific sequences, the 0.6-kilobase (kb) *Bam*HI-*Eco*RI *env* gene fragment of F-SFFV (BE fragment) cloned into pBR322 was used (11, 32). This plasmid was generously provided by D. Linemeyer and E. Scolnick, National Cancer Institute, Bethesda, Md.

**Analysis of leukemogenicity.** SFFV pathogenesis was assayed by previously described methods (5, 17 37, 44). The wild-type and mutant R-SFFVs, rescued from nonproducer cell lines by superinfection with R-MuLV, were injected into the tail veins of at least six different 4- to 6-week-old female NIH/Swiss mice. Control mice were uninfected or infected with R-MuLV alone. Spleens were examined after 14, 21, and 28 days for macroscopic foci (5) and for enlargement (44). Spleens not showing gross evidence of disease were examined histologically (17, 37). To verify that the apparently weak leukemogenicity of these mutant R-SFFVs was due to decreases in leukemogenic potential and not due to low titers of virus injected, virus was amplified in vivo by serial passage in NIH/Swiss mice. Spleens were removed from infected mice 14 days after infection and were subsequently pooled, homogenized, and extracted with cold phosphate-buffered saline. Filtrates of these spleen homogenates were then injected into fresh mice. This in vivo serial passaging of virus in NIH/Swiss mice was conducted for a total of four passages and the extent of R-SFFV-specific disease was examined at each stage of the amplification procedure.

## RESULTS

**Mutant R-SFFV clones 3-25 and 4-3 encoding abnormally sized gp54-related glycoproteins with apparent $M_r$s of 52,000 (gp52) and 45,000 (gp45), respectively.** Figure 1 shows an electrophoretic analysis of L-[$^{35}$S]methionine-labeled viral gene products synthesized in cell lines clonally infected with mutant or wild-type R-SFFVs. As shown in this figure and as described previously (48), wild-type R-SFFV encodes an *env* gene product with an apparent $M_r$ of 54,000 (gp54) (Fig. 1, lane 8) and *gag* gene products (lane 7) which appear to be electrophoretically indistinguishable from those encoded by ecotropic R-MuLV. Consistent with previous studies (48), the gp54 molecule can be precipitated either with monospecific antiserum prepared against MuLV gp70 (Fig. 1, lane 8) or with a rat antiserum that recognizes a determinant found in the gp70s of dual-tropic, but not ecotropic, MuLVs (lane 9). The clone 4-3 (Fig. 1, lanes 1 through 3) and clone 3-25- (lanes 4 through 6) infected cell lines synthesize antigenically

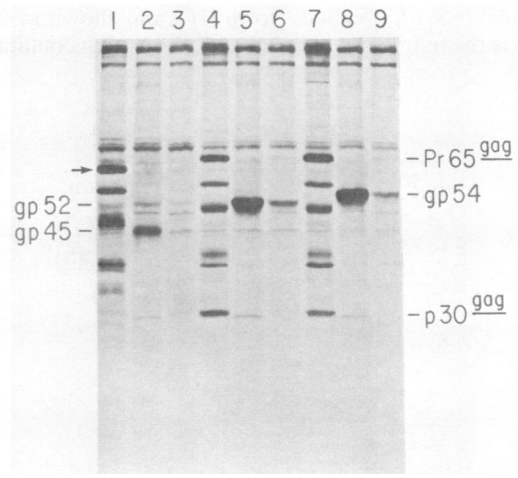


FIG. 1. Electrophoretic analysis of L-[$^{35}$S]methionine-labeled proteins encoded by wild-type and mutant R-SFFVs. Cell lines infected with mutant R-SFFV clone 4-3 (lanes 1 through 3) or 3-25 (lanes 4 through 6) or with wild-type R-SFFV (lanes 7 through 9) were pulse-labeled by incubation with 100 μCi of L-[$^{35}$S]methionine for 2 h at 37°C. The cells were subsequently lysed, and the viral-encoded proteins in the lysates were immunoprecipitated with monospecific serum to p30$^{gag}$ (lanes 1, 4, and 7) or gp70$^{env}$ (lanes 2, 5, and 8) or with a rat serum that recognizes SFFV-specific antigenic determinants that occur on gp54 (lanes 3, 6, and 9). Immunoprecipitated proteins were electrophoresed in 10 to 20% polyacrylamide gels containing 0.1% sodium dodecyl sulfate.

related *env* gene products with apparent $M_r$s of 45,000 (lane 2, gp45) and 52,000 (lane 5, gp52), respectively. Both glycoproteins, gp45 and gp52, are strongly precipitated with monospecific antiserum prepared against ecotropic MuLV gp70 (Fig. 1, lanes 2 and 5) and, in the case of the latter molecule, with the rat antiserum that recognizes the gp70s of dual-tropic MuLVs (lane 6). Although gp45 does not appear to be precipitated with the rat antiserum in Fig. 1 (lane 3), extensive overexposure of the fluorogram does reveal the weak precipitation of this molecule by this antiserum.

The mutant R-SFFV clone 3-25 encodes *gag* gene products which appear to be electrophoretically indistinguishable from those encoded by wild-type R-SFFV (compare Fig. 1, lanes 4 and 7). These include the well-characterized *gag* polyprotein Pr65 and its cleavage products, including p30. On the contrary, *gag* gene products synthesized in the clone 4-3-infected cell line (Fig. 1, lane 1) are abnormal. The *gag* component with an $M_r$ of 62,000 detected in the clone 4-3-infected cell line (see Fig. 1, arrow) is rapidly labeled with L-[$^{35}$S]methionine and can be immunoprecipitated with monospecific antisera prepared against each of the four MuLV *gag* core proteins, p15, p12, p30, and p10 (C. Machida and D. Kabat, unpublished data). Furthermore, this component with an $M_r$ of 62,000 is only partially processed by proteolysis (see Fig. 1, lane 1). Consistent with evidence described below, these mutant phenotypes were readily transferred with rescued virus into uninfected cell lines.

**Mutant R-SFFV clone 3-25 and 4-3 nucleic acids containing additional and deleted genetic sequences, respectively.** Figure 2 shows a Southern blot analysis of unintegrated proviral DNA extracted from NIH-3T3 cells 24 h after infection with wild-type or mutant virus preparations. The blot was hybridized with a $^{32}$P-labeled SFFV-specific *env* gene (BE fragment) hybridization probe. The SFFV-related proviral nucleic acids were readily detected, confirming evidence described above that these mutant SFFVs can be packaged into released infectious virus particles. The major R-SFFV component (8.3 kb; called "form III" and shown in Fig. 2, lane 2) is the unintegrated linear provirus which contains two

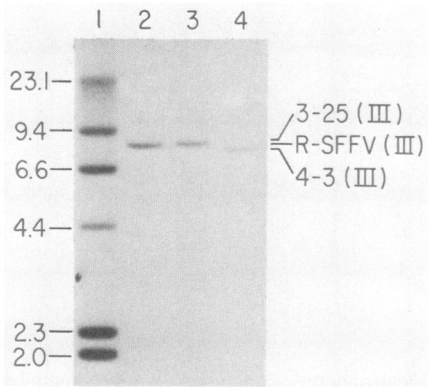


FIG. 2. Southern blot analysis of unintegrated proviral DNA from cells infected with either wild-type R-SFFV (lane 2) or mutant R-SFFVs (clones 3-25 and 4-3 in lanes 3 and 4, respectively). Infection conditions are described in the text. DNA (5 to 20 μg) from the Hirt supernatants was electrophoresed in a horizontal 0.75% agarose gel before blotting to diazophenylthioether paper. The blot was hybridized to $^{32}$P-labeled SFFV-specific fragment BE probe. Lane 1 contains radioactive wild-type lambda DNA after digestion with *Hin*dIII (sizes indicated are in kilobases). Lambda standards were electrophoresed on both sides of the gel to aid in distinguishing the small size differences in proviral DNAs.

long-terminal repeats. Careful electrophoretic size comparisons of the proviral DNAs show that the clone 3-25 DNA (8.35 kb; Fig. 2, lane 3) is slightly larger than the wild-type molecule (8.3 kb) and that the clone 4-3 DNA (8.2 kb; lane 4) is slightly smaller. Longer autoradiographic exposures reveal comparable size differences between wild-type and mutant R-SFFV form I DNAs (supercoils containing one or two long-terminal repeats) (C. Machida, R. Bestwick, and D. Kabat, unpublished data).

A detailed restriction map of wild-type R-SFFV has been established by using both unintegrated proviral DNA and a molecular clone of circular unintegrated proviral DNA (R. Bestwick and D. Kabat, manuscript in preparation). From this map information a set of restriction enzymes were chosen that allowed localization of the mutation sites to either the 3′ or 5′ half of the *env* gene. A partial restriction map, illustrating sites useful in localizing the mutation sites contained in 3-25 and 4-3 proviral DNA, is shown in Fig. 3 (panel C). Panels A and B are Southern blot analyses of unintegrated wild-type and mutant proviral DNAs digested with either one or two restriction endonucleases. Both blots were hybridized with a $^{32}$P-labeled SFFV-specific fragment BE hybridization probe. R-SFFV proviral DNA, when digested with *Bam*HI plus *Eco*RI (Fig. 3, panel A, lane 2), *Bam*HI alone (panel A, lane 3), or *Hin*dIII alone (panel A, lane 4), produce overlapping *env* gene fragments with sizes of 0.60, 1.95, and 2.65 kb, respectively. Digestion of mutant 3-25 DNA (Fig. 3A, lanes 5 through 7) with the same battery of restriction endonucleases generates fragments (0.65, 2.0, and 2.7 kb, respectively) that are all consistently and reproducibly larger by a small amount (ca. 50 base pairs [bp]) than those produced from the wild-type molecule. These observations are consistent with the hypothesis that the mutant 3-25 proviral DNA contains additional genetic sequences located in the 600-bp *Bam*HI-*Eco*RI *env* gene region. Similar analyses comparing wild-type and 4-3 proviral DNA (Fig. 3, panel B) reveals reproducible size differences only between the *Bam*HI-*Kpn*I *env* gene fragments (1.8 kb for wild-type R-SFFV DNA, lane 3, and 1.7 kb for 4-3 DNA, lane 6). Reproducible size differences were not observed between wild-type and 4-3 *env* gene fragments generated by digestion with either *Bam*HI plus *Eco*RI (0.60-kb fragments; Fig. 3B, lanes 1 and 4, respectively) or *Hin*dIII plus *Eco*RI (1.1-kb fragment; lanes 2 and 5, respectively). These observations have localized deleted sequences in 4-3 DNA to a region extending from the *Eco*RI site to the *Kpn*I site in the 3′ long-terminal repeat. Northern blot and protein fragmentation evidence described below is consistent with these results and further localizes the deletion to the 3′ half of the *env* gene.

Moreover, polyadenylic acid-containing RNAs were extracted from infected cell lines and were analyzed by the Northern blot technique with the $^{32}$P-labeled SFFV-specific fragment BE hybridization probe. As shown in Fig. 4 and as described previously (8), the R-SFFV genomic and subgenomic RNAs have sizes of 7.8 and 2.1 kb, respectively. Consistent with the size determinations of the mutant proviral DNAs, the subgenomic *env* mRNAs of mutants 3-25 and 4-3 were found to be slightly larger (2.15 kb; Fig. 4, lane 2) and slightly smaller (2.0 kb; lane 3), respectively, than that observed for the wild-type *env* mRNA.

**Protein fragmentation patterns indicating that the mutations affect nonoverlapping domains of the glycoprotein.** The gp54 molecule encoded by wild-type R-SFFV is cleaved by *S. aureus* V8 protease to form primary fragments V-1 ($M_r$, 33,500) and V-2 ($M_r$, 19,300) (Fig. 5, lane 2). By increasing

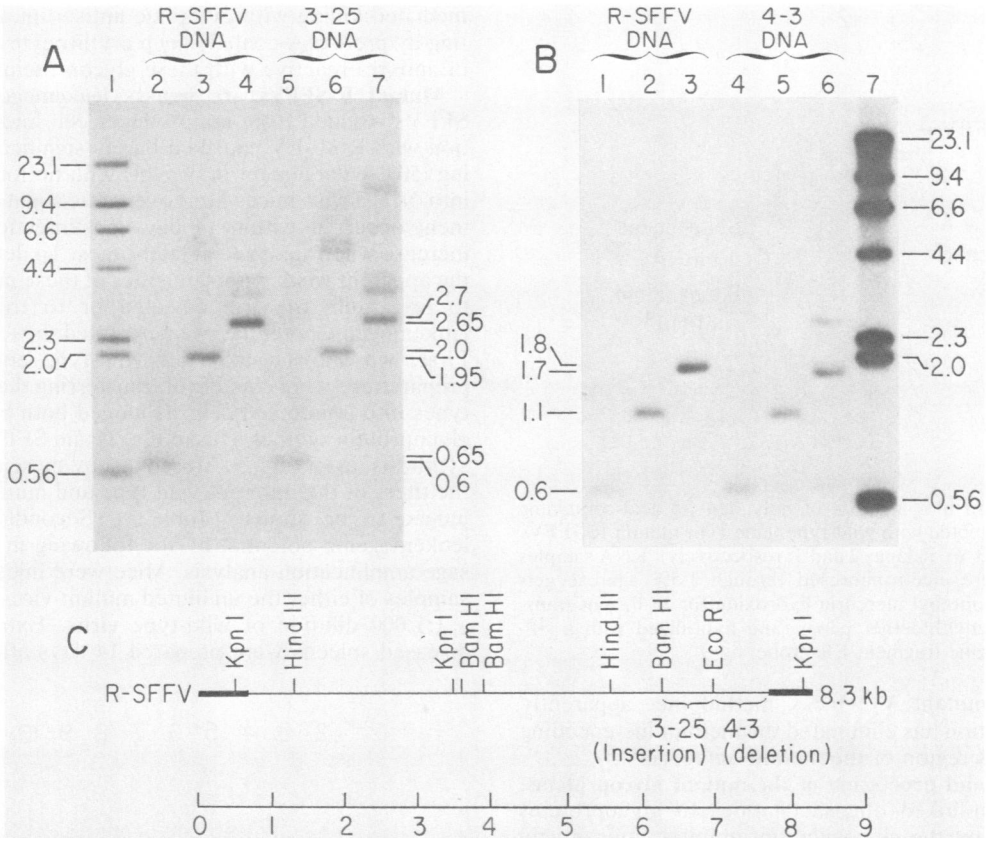


FIG. 3. Restriction endonuclease analysis of unintegrated proviral DNA from cells infected with wild-type (panel A, lanes 2 through 4 and panel B, lanes 1 through 3) or mutant (clone 3-25, panel A, lanes 5 through 7 and clone 4-3, panel B, lanes 4 through 6) R-SFFVs. DNA (5 to 20 μg) from Hirt supernatants was digested with *Eco*RI plus *Bam*HI (panel A, lanes 2 and 5 and panel B, lanes 1 and 4), *Bam*HI alone (panel A, lanes 3 and 6), *Hin*dIII alone (panel A, lanes 4 and 7), *Hin*dIII plus *Eco*RI (panel B, lanes 2 and 5) or *Bam*HI plus *Kpn*I (panel B, lanes 3 and 6) for 60 min at 37°C and electrophoresed in a horizontal 0.75% agarose gel before being blotted with diazophenylthioether paper. The blot was hybridized to $^{32}$P-labeled SFFV-specific fragment BE probe. Lane 1 in panel A and lane 7 in panel B contain radioactive wild-type lambda DNA after digestion with *Hin*dIII. Lambda standards were electrophoresed on both sides of all gels to distinguish small size differences in restriction fragments. Panel C shows a partial restriction map of R-SFFV and locations of *env* gene mutations in 3-25 and 4-3 proviral DNAs.

the amount of V8 enzyme used to digest the gp54 molecule, V-1 is further cleaved to produce secondary fragments V-3 ($M_r$, 20,000) and V-4 ($M_r$, 12,400) (Fig. 5, lane 3), and tertiary fragments are observed when the protease concentration is further increased (lane 4).

The amino- and carboxyl-terminal orientations of the V-1 and V-2 fragments have been analyzed by the following approaches. First, the immunoprecipitation of V-1 (but not V-2) occurs with a rat antiserum (45, 58) that recognizes a determinant found in the amino-terminal region of gp70s encoded by dual-tropic but not ecotropic MuLVs. Second, restriction endonuclease analyses described above suggest that genetic alterations have occurred in the 5′ or 3′ halves of the 3-25 and 4-3 *env* genes, respectively. Third, brief pulse labeling (5, 10, 15, and 30 min) of R-SFFV gp54 with L-[$^{35}$S]methionine, followed by V8 digestion of the isolated glycoprotein to yield only the primary fragments shows a preferential labeling of V-2 compared with V-1 at the shorter labeling times. All of these results support an amino- and carboxyl-terminal location for the V-1 and V-2 fragments, respectively.

In a previous report (46), the gp55 encoded by wild-type F-SFFV was shown to be cleaved by *S. aureus* V8 protease to form primary fragments V-1 ($M_r$, 32,500) and V-2 ($M_r$, 21,500). Although we originally concluded that V-2 of F-SFFV was not labeled with L-[$^{35}$S]methionine, recent findings reveal a weak labeling of this V-2. Like V-1 derived from R-SFFV gp54, V-1 from F-SFFV gp55 contains the antigenic site(s) specifically related to the gp70s of dual-tropic MuLVs.

Figure 5 also shows comparative cleavage analyses of L-[$^{35}$S]methionine-labeled glycoproteins encoded by mutant R-SFFVs (3-25 gp52, lanes 5 through 8; 4-3 gp45, lanes 9 through 12). The V-1 fragment of gp52 (V-1$^{3\text{-}25}$; $M_r$, 31,600) (Fig. 5, lane 6) is electrophoretically different from V-1 obtained from gp54 (lane 2). The apparent size difference between these V-1 fragments (1.9 kilodaltons) is not significantly different than the size difference observed between the intact mutant and wild-type glycoproteins. Furthermore, the abnormality in the gp52 molecule was more precisely localized to the V-3 subregion (V-3$^{3\text{-}25}$; $M_r$, 18,000) of the V-1 domain. In contrast, the mutant gp45 molecule appears to contain an intact V-1 domain (Fig. 5, lane 10). Secondary cleavage fragments V-3 and V-4 generated from this V-1 are electrophoretically identical to the corresponding fragments obtained from wild-type gp54. Clearly, the abnormality in the clone 4-3 *env* glycoprotein is located within the carboxyl-terminal V-2 domain. Unlike V-2 derived from the wild-type

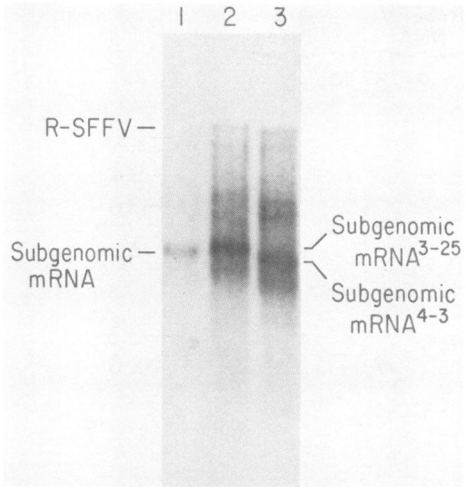


FIG. 4. Northern blot analysis of polyadenylic acid-containing RNA from cells infected with wild-type (lane 1) or mutant R-SFFVs (clones 3-25 and 4-3 are in lanes 2 and 3, respectively). RNA samples (10 to 20 μg) were electrophoresed through 1.5% agarose gels containing 10 mM methyl mercuric hydroxide for 18 h, blot transferred to diazophenylthioether paper, and hybridized with a $^{32}$P-labeled SFFV-specific fragment BE probe.

molecule, this mutant V-2 lacks methionine, apparently because the deletion has eliminated the methionine-encoding portion from this region of the mutant *env* gene.

**Glycosylation and processing of the mutant glycoproteins.** Addition of Asn-linked oligosaccharides to glycoproteins can be blocked by the glycosylation inhibitor tunicamycin (29, 53). However, the resulting non-glycosylated proteins are often susceptible to intracellular proteolysis and such susceptibility can be enhanced by mutations (34, 41). The polypeptide chain encoded by the *env* gene of wild-type R-SFFV has an apparent $M_r$ of 44,000 (Fig. 6, lanes 2 and 8). Consistent with nucleic acid evidence described above, the *env*-related polypeptide chain encoded by mutant 4-3 is smaller ($M_r$, 38,000; Fig. 6, lane 4). Although the reduced size of gp45 appears to be caused primarily by a shortening of its polypeptide chain, it is possible that this molecule may also be deficient in asparagine-linked oligosaccharides. Similar studies indicated that the polypeptide chain of gp52 (Fig. 6, lane 6) is at least equivalent in size to the polypeptide chain of wild-type gp54 (lane 8). However, this size difference was especially difficult to analyze because the non-glycosylated mutant component was very rapidly degraded. Conceivably, the polypeptide detected may be a degradation intermediate rather than the complete polypeptide chain.

Previous studies have shown that a small proportion of the *env* glycoprotein encoded by F-SFFV and R-SFFV occurs on the surfaces of infected cells, whereas the majority occurs in organelles with the buoyant density of the rough endoplasmic reticulum (47, 48). Because only an extremely small proportion (an estimated 0.5%) of the R-SFFV gp54 component occurs on the cell surface (48), it has been very difficult to determine whether the mutant and wild-type glycoproteins differ significantly in this respect. Nevertheless, our preliminary evidence implies that the mutant glycoproteins do not reach the cell surface. Specifically, in contrast to our previously described analyses of gp54 (48), corresponding larger, heterogeneously sialylated, [$^3$H]glucosamine-labeled components of gp52 and gp45 were not detected. Furthermore, plasma membrane forms of gp52 and gp45 were also not detected by iodination with [$^{125}$I]iodine, by complement-mediated killing with cytotoxic antiserum, or by cell rosetting by protein A-coated sheep erythrocytes in the presence of antisera reactive with these glycoproteins.

**Mutant R-SFFVs are weakly leukemogenic.** Mutant R-SFFVs rescued from nonproducer cell lines by superinfection with R-MuLV caused a barely significant spleen swelling (50% enlargement in weight) with no foci when injected into NIH/Swiss mice. Moreover, this slight splenic enlargement occurred within 14 days postinfection, and did not increase when analyzed at later times. To determine whether the apparent weak pathogenicities of these mutants were due to low titers of virus injected or to true decreases in leukemogenic potential, we performed the following studies. First, we showed that our wild-type and mutant virus preparations were capable of transferring their SFFV phenotypes into uninfected cells as judged both by studying viral glycoprotein synthesis (as in Fig. 1) and SFFV proviral DNA synthesis (as in Fig. 2). Moreover, by these transfer criteria, the titers of the different wild-type and mutant viruses were judged to be similar (Table 1). Secondly, we analyzed leukemogenic potential by the following in vivo serial passage amplification analysis. Mice were injected with 0.5-ml samples of either the undiluted mutant virus preparations or a 1:1,000 dilution of wild-type virus. Extracts of pooled, diseased spleens were prepared 14 days after infection and

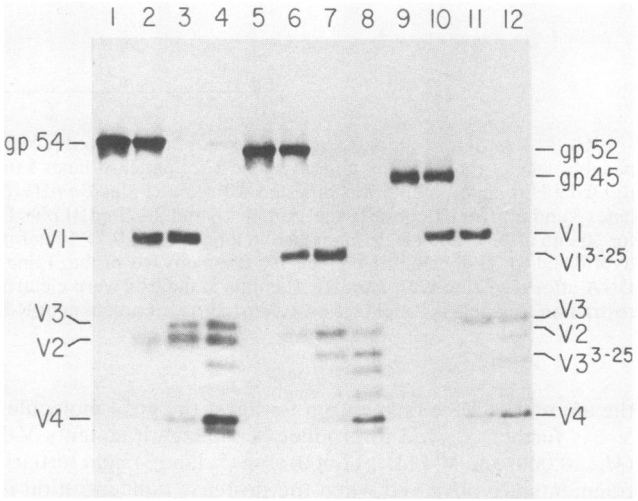


FIG. 5. Proteolytic fragmentation of glycoproteins encoded by wild-type (lanes 1 through 4) or mutant R-SFFVs (clone 3-25, lanes 5 through 8; clone 4-3, lanes 9 through 12). The L-[$^{35}$S]methionine-labeled glycoproteins were eluted from polyacrylamide gels as described in the text and were subjected to proteolysis for 30 min at 37°C with increasing amounts (0, 0.1, 1.0, and 10.0 μg) of *S. aureus* V8 protease. The digests were then re-electrophoresed, and the radioactive components were then visualized by fluorography. V-1 and V-2 are the primary proteolytic fragments (apparent $M_r$s, 33,500 and 19,300, respectively). V-3 and V-4 are secondary cleavage fragments (apparent $M_r$s, 20,000 and 12,400, respectively) derived entirely from V-1. V-1$^{3-25}$ and V-3$^{3-25}$ represent fragments obtained from clone 3-25 gp52. Careful electrophoretic comparisons of V-2 fragments derived from R-SFFV gp54 and 3-25 gp52 show no reproducible differences between the sizes of these two molecules. The gp45 encoded by mutant 4-3 is abnormal in V-2 (V-2$^{4-3}$, $M_r$, 12,500). This is based partly on apparent identity of its V-1 with the V-1 from gp54. V-2 from gp45 apparently lacks methionine. The estimation of its size is based on data obtained from gp45 labeled with a mixture of $^3$H-amino acids. The band in lane 12 that migrates at the same apparent $M_r$ as V-2 is a tertiary cleavage fragment derived from V-3.

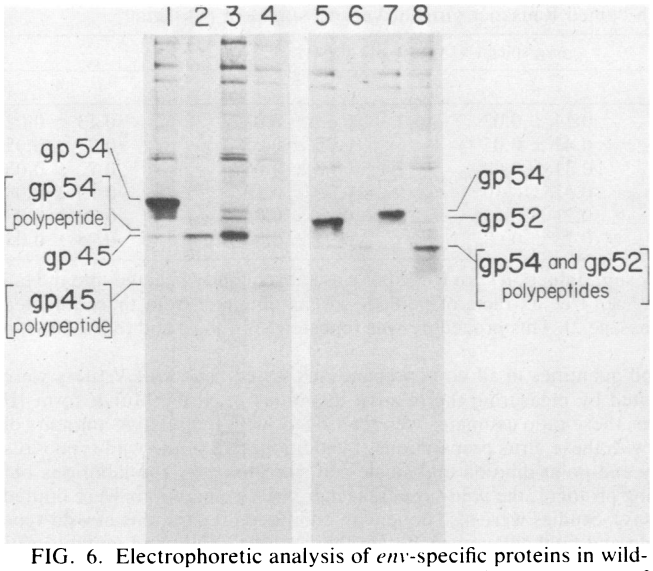


FIG. 6. Electrophoretic analysis of *env*-specific proteins in wild-type and mutant R-SFFV-infected cell lines grown in the presence of the glycosylation inhibitor tunicamycin. Cell lines infected with wild-type R-SFFV (lanes 2 and 8) or with mutant R-SFFV clones 4-3 (lane 4) or 3-25 (lane 6) were treated with 25 μg of tunicamycin per ml both before (3 h) and during labeling (10 min) with 100 μCi of L-[$^{35}$S]methionine. These cultures, as well as labeled control cultures incubated without tunicamycin (R-SFFV-infected cells, lanes 1 and 7; clone 4-3- and 3-25-infected cells, lanes 3 and 5, respectively), were lysed after labeling and the viral proteins in the lysates were immunoprecipitated with anti-gp70$^{env}$ serum. Immunoprecipitated proteins were electrophoresed in 10 to 20% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Designations on the left and right sides of the figure refer to molecules in either lanes 1 through 4 or 5 through 8, respectively.

were subsequently serially injected into previously unused animals for a total of four passages. The splenic pathology was analyzed for each of the virus samples at each stage of the amplification procedure. Even small amounts of wild-type R-SFFV at dilutions too low to cause rapid splenomegaly became extensively amplified by serial passaging, and they caused massive splenomegaly with extensive focal development within 14 days after infection by the second transfer (Table 1). On the contrary, the diseases caused by clone 3-25 and 4-3 mutant viruses became only slightly amplified, even after extensive serial passaging. Nevertheless, these results indicate that the mutant R-SFFVs replicate in vivo, and we confirmed this by reisolating the mutant 3-25 virus from a mouse spleen after four serial in vivo passages. The *env* gene products of the original and reisolated 3-25 viruses have identical apparent $M_r$s and V8 protease fragmentation patterns. Histological examination of mouse spleens after four serial passages with the mutant viruses confirmed the diagnosis of a mild Rauscher-like erythroproliferative disease.

## DISCUSSION

**Mutant R-SFFVs.** In this paper we describe two spontaneous weakly leukemogenic mutants of R-SFFV which encode abnormal gp54-related glycoproteins. Both of these mutant R-SFFVs are transmissible, since their phenotypes and genotypes can be readily transmitted with rescued virus between cells (e.g., Fig. 2) and in vivo (Table 1). This data is in agreement with previously described evidence that F-SFFV transmission, packaging, and replication functions are not affected by *env* gene mutations (46).

One of these mutants, clone 3-25, contains an insertion of ca. 50 bp in the 600-bp *Bam*HI-*Eco*RI region of its *env* gene (Fig. 3). Moreover, this mutation affects the V-3 subregion of the V-1 domain (Fig. 5) and causes the loss of an asparagine-linked oligosaccharide attachment site in the encoded polypeptide chain (Fig. 6). Because of its reduced glycosylation, 3-25 gp52 is smaller than R-SFFV gp54. The orientation of these DNA and protein fragments provides consistent evidence because the 600-bp *Bam*HI-*Eco*RI DNA fragment derives from the 5′ side of the 1,400-bp *env* gene (2), and the V-1 glycoprotein domain contains antigens related to the amino-terminal regions of the gp70s of dual-tropic MuLVs (40, 58).

The second R-SFFV mutant, clone 4-3, appears to contain mutations in both its *gag* and *env* genes (Fig. 1). Northern (Fig. 4) and Southern (Fig. 2 and 3) blot analyses and gp45 fragmentation studies (Fig. 5) indicate that the 4-3 *env* gene contains a deletion of ca. 100 bp which affects the carboxyl-terminal V-2 domain of the encoded glycoprotein (Fig. 5). Although gp45 contains an intact V-1 domain, the intact glycoprotein reacts only weakly with the rat antiserum (Fig. 1). Presumably, the antigenic site(s) may be blocked in the folded gp45 molecule. By these criteria, the 3-25 and 4-3 mutants have abnormalities in nonoverlapping portions of their *env* genes.

**Reduced leukemogenicity of R-SFFV mutants.** Our studies provide evidence that the two R-SFFV mutants cause a significant but strongly reduced splenic erythroblast hyperplasia compared with wild-type R-SFFV (e.g., Table 1). Moreover, their weakened pathogenesis is highly significant because the R-SFFV mutants are transmissible (e.g., Fig. 2) and because they replicate in vivo and can be serially transferred between mice (Table 1). Therefore, the fact that their disease does not become amplified to the wild-type level by serial passaging strongly implies that the mutant R-SFFVs have a reduced pathogenic activity. Even low titers of wild-type Rauscher virus which do not initially cause detectable spleen enlargement become amplified by serial passaging and then cause massive splenomegaly within 14 days of injection (Table 1).

There are several reasons for inferring that the reduced pathogenicity of these R-SFFV mutants may be a consequence of their *env* gene mutations. First, the clone 3-25 mutant apparently contains an intact *gag* gene indistinguishable from that of wild-type R-SFFV (Fig. 1) or R-MuLV (48). On the contrary, the clone 4-3 mutant also contains a mutation in its *gag* gene (Fig. 1). Second, in parallel with the isolation of these two R-SFFV *env* mutants, we isolated numerous clones of wild-type R-SFFV with apparently intact *env* genes. All 10 of those which were isolated and tested in this study, and 10 other cloned gp54-encoding R-SFFVs described in an earlier report (48) were highly pathogenic. Consequently, the hypothesis that loss of pathogenesis cosegregates with R-SFFV *env* gene abnormality is strongly supported (probability greater than 99%). Third, we have recently described the isolation and characterization of three *env* gene mutants of F-SFFV, and all of these are also either non-leukemogenic or weakly leukemogenic (46). In agreement with this report, the latter mutants also contain abnormalities affecting non-overlapping domains of the envelope glycoprotein. Considered together, this evidence suggests that the entire folded membrane glycoprotein may be involved in pathogenesis rather than merely a fragment of the gene or of the encoded glycoprotein.

TABLE 1. Analysis of spleen weights of mice infected with cloned Rauscher virus at various stages of passaging[a]

| Virus inoculated[b] | Avg spleen wt (g)[c] ± SD at passage: | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| R-MuLV[d] | 0.12 ± 0.02 | 0.14 ± 0.02 | 0.13 ± 0.03 | 0.13 ± 0.02 |
| Wild-type R-SFFV (clone 3-32) | 0.12 ± 0.01 | 0.47 ± 0.05 | 0.75 ± 0.07 | 0.71 ± 0.05 |
| Wild-type R-SFFV (RV NIH clone 6) | 0.13 ± 0.02 | 0.31 ± 0.08 | 0.68 ± 0.06 | 0.72 ± 0.06 |
| Wild-type R-SFFV (RV NIH clone 18) | 0.14 ± 0.03 | 0.51 ± 0.10 | 0.75 ± 0.10 | 0.75 ± 0.08 |
| Mutant R-SFFV[e] (clone 3-25) | 0.12 ± 0.02 | 0.21 ± 0.03 | 0.28 ± 0.05 | 0.29 ± 0.05 |
| Mutant R-SFFV[e] (clone 4-3) | 0.12 ± 0.02 | 0.23 ± 0.03 | 0.24 ± 0.05 | 0.25 ± 0.05 |

[a] As described in the text, virus rescued from R-SFFV-infected cells by superinfection with R-MuLV was subsequently injected into the tail veins of 10 4- to 6-week-old female NIH/Swiss mice. Two weeks later, cell-free extracts of pooled spleens obtained from these infected animals were prepared and then injected into previously unused mice (passage 2). This procedure was repeated for a third and fourth in vivo passage.

[b] The SFFV and MuLV components were present in similar ratios and quantities in all virus preparations tested. The MuLV titers were measured by the S+L− method. The SFFV/MuLV ratios were estimated by measuring the relative quantities of SFFV/MuLV form III proviruses 30 hours after infection of NIH-3T3 fibroblasts (46). Moreover, these ratio estimates were consistent with the relative amounts of SFFV and MuLV encoded glycoproteins formed in cells newly infected with these virus preparations. In addition, two of the wild-type virus preparations (RV NIH clone 6 and RV NIH clone 18) were titrated by end-point dilution and single-cell subcloning by the laborious but accurate procedure previously described (45, 49). For this serial passaging protocol, the wild-type Rauscher virus preparations were diluted 1,000-fold to contain ca. $10^2$ R-MuLV per ml (as estimated by S+ L− assay). Studies were also done with undiluted preparations of wild-type Rauscher virus. The undiluted virus preparations caused extremely rapid and fatal splenomegaly. On the contrary, undiluted mutant virus preparations containing ca. $10^5$ R-MuLV and mutant R-SFFV per ml were used for this study. This was done to allow the mutant viruses an opportunity to become maximally amplified. A 0.5-ml amount of all virus preparations was then used for the initial injections.

[c] Number of mice analyzed per sample at each passage: 1, 10 mice; 2, 6 mice; 3, 4 mice; and 4, 4 mice.

[d] Parallel studies indicated that spleens obtained from uninfected mice were identical in both size and macroscopic appearance to those obtained from mice infected with our cloned R-MuLV (C. Machida and D. Kabat, unpublished data).

[e] Histological examination of mouse spleens after 4 serial passages with the mutant R-SFFVs confirmed the diagnosis of a mild Rauscher-like erythroproliferative disease. Spleen touch impressions and histological sectioning showed the presence of increased numbers of erythroid precursors in spleens obtained from mutant R-SFFV-infected mice.

Consequently, we suggest that the R-SFFV-encoded gp54 glycoprotein is required for inducing the erythroid cell hyperplasia which characterizes the early preleukemic phase of Rauscher disease. This conclusion needs to be qualified in several ways. First, we have analyzed these mutants only by established gross and histological criteria in 4 to 6-week-old NIH/Swiss female mice. Conceivably, these mutants might cause a more pronounced disease or even a different disease in other murine strains, in newborn mice, or with a longer latency in nonerythroid hematopoietic tissues. We are currently studying these possibilities. Second, it seems obvious that other regions of the SFFV genome would also be required for pathogenesis, including the long terminal repeats and other regions required for virus transmission. Nevertheless, nonpathogenic mutants with apparently normal *env* genes have not been detected among the relatively small number of cloned viruses which we have analyzed. Third, it is apparent that the two R-SFFV mutants are weakly pathogenic and that they cause a rapid splenic erythroproliferative disorder. Therefore, pathogenic activity of R-SFFV is not completely eliminated by at least certain substantial *env* gene abnormalities. This is most striking in the case of the clone 4-3 mutant which encodes gp45. The latter glycoprotein lacks a substantial proportion of the carboxyl-terminal V-2 domain. Consequently, we propose that only a portion of gp54 is directly required for stimulation of erythroblast proliferation and that the remainder may perform an ancillary function such as glycoprotein stabilization or facilitation of proper intracellular placement.

**Progressive leukemogenesis.** It has been proposed that the SFFVs and dual-tropic MuLVs are structurally related members of a single virus class which can cause diverse progressive neoplasms by common mechanisms (8, 10, 13, 20, 54). In general, these viruses cause premalignant tissue-specific proliferation of hematopoietic stem cells with subsequent formation of an increasingly malignant subclone(s) capable of indefinite proliferation in secondary recipients or in culture (35, 57). Our results and related earlier studies (31, 33, 46) suggest that the SFFV *env* genes are oncogenes which initiate this process of progressive leukemogenesis. Such initiating or causal oncogenes may be more relevant for understanding natural progressive tumorigenesis than the previously described oncogenes which seem to often be activated at late or final stages of tumorigenic development (15, 21, 23).

## ACKNOWLEDGMENTS

We are grateful to Ethel Polonoff and Bruce Boswell for helpful discussions and expert technical assistance. We are also grateful to Martin Ruta who participated in the initial isolation of SFFV mutants in this laboratory.

This research was supported by Public Health Service grant CA25810 and in part by American Cancer Society grant MV-160.

## LITERATURE CITED

1. **Alwine, J. C., D. J. Kemp, B. A. Parker, J. Reiser, J. Renart, G. R. Stark, and G. M. Wahl.** 1979. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. Methods Enzymol. **68**:220–242.
2. **Amanuma, H., A. Katori, M. Obata, N. Sagata, and Y. Ikawa.** 1983. Complete nucleotide sequence of the gene for the specific glycoprotein (gp55) of Friend spleen focus-forming virus. Proc. Natl. Acad. Sci. U.S.A. **80**:3913–3917.
3. **Anand, R., and R. Steeves.** 1980. How many types of erythroleukaemia are induced by retroviruses in mice? Nature (London) **286**:615–617.
4. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. **69**:1408–1412.
5. **Axelrad, A. A., and R. A. Steeves.** 1964. Assay for Friend leukemia virus: rapid quantitative method based on enumeration of macroscopic spleen foci in mice. Virology **24**:513–518.

6. **Bailey, J. M., and N. Davidson.** 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. **70**:75–85.
7. **Bassin, R. H., N. Tuttle, and P. J. Fishinger.** 1971. Rapid cell cultures assay technique for murine leukemia viruses. Nature (London) **229**:564–566.
8. **Bestwick, R., M. Ruta, A. Kiessling, C. Faust, D. Linemeyer, E. Scolnick, and D. Kabat.** 1983. Genetic structure of Rauscher spleen focus-forming virus. J. Virol. **45**:1217–1222.
9. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
10. **Bosselman, R. A., F. van Straaten, C. Van Beveren, I. M. Verma, and M. Vogt.** 1982. Analysis of the *env* gene of a molecularly cloned and biologically active Moloney mink cell focus-forming proviral DNA. J. Virol. **44**:19–31.
11. **Chattopadhay, S. K., M. W. Cloyd, D. L. Linemeyer, M. R. Lander, F. Rands, and D. R. Lowy.** 1982. Origin and role of mink cell focus-forming viruses in murine thymic lymphomas. Nature (London) **295**:25–31.
12. **Cleveland, D., S. Fischer, M. Kirschner, and U. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
13. **Cloyd, M. W.** 1983. Characterization of target cells for MCF viruses in AKR mice. Cell **32**:217–225.
14. **Cloyd, M. W., J. W. Hartley, and W. P. Rowe.** 1980. Lymphomagenicity of recombinant cell focus-inducing murine leukemia viruses. J. Exp. Med. **151**:542–552.
15. **Cooper, G. M., and Neiman, P. E.** 1981. Two distinct candidate transforming genes of lymphoid leukosis virus-induced neoplasms. Nature (London) **292**:857–858.
16. **Dawson, P. J., W. M. Rose, and A. H. Fieldsteel.** 1966. Lymphatic leukemia in rats and mice inoculated with Friend virus. Br. J. Cancer **20**:114–121.
17. **Dresler, S., M. Ruta, M. J. Murray, and D. Kabat.** 1979. Glycoprotein encoded by the Friend spleen focus-forming virus. J. Virol. **30**:564–575.
18. **Elder, J. H., J. W. Gautsch, F. C. Jensen, R. A. Lerner, J. W. Hartley, and W. P. Rowe.** 1977. Biochemical evidence that MCF murine leukemia viruses are envelope (env) gene recombinants. Proc. Natl. Acad. Sci. U.S.A. **74**:4676–4680.
19. **Evans, L. H., S. Dresler, and D. Kabat.** 1977. Synthesis and glycosylation of polyprotein precursors to the internal core proteins of Friend murine leukemia virus. J. Virol. **24**:865–874.
20. **Evans, L., M. Nunn, P. Duesberg, D. Troxler, and E. Scolnick.** 1980. RNAs of defective and nondefective components of Friend anemia and polycythemia virus strains identified and compared. Cold Spring Harbor Symp. Quant. Biol. **44**:823–836.
21. **Fidler, I. J., and I. R. Hart.** 1982. Biological diversity in metastatic neoplasms: origins and implications. Science **217**:998–1003.
22. **Fieldsteel, A. H., P. J. Dawson, and C. Kurahara.** 1969. Induction of lymphatic leukemia in Balb/c mice from the original isolate of Rauscher virus. Br. J. Cancer **23**:806–812.
23. **Foulds, L.** 1969. Neoplastic development, vol. 1, p. 41–86. Academic Press, London.
24. **Haas, M., and V. Patch.** 1980. Cell-surface antigens associated with dualtropic and thymotropic murine leukemia viruses inducing thymic and nonthymic lymphomas. J. Exp. Med. **151**:1321–1333.
25. **Hankins, D., and D. Troxler.** 1980. Polycythemia and anemia inducing erythroleukemia viruses exhibit differential erythroid transforming effects in vitro. Cell **22**:693–699.
26. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
27. **Kabat, D., M. Ruta, M. J. Murray, and E. Polonoff.** 1980. Immunoselection of mutants deficient in cell surface glycoproteins encoded by murine erythroleukemia viruses. Proc. Natl. Acad. Sci. U.S.A. **77**:57–61.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
29. **Leavitt, R., S. Schlesinger, and S. Kornfeld.** 1977. Tunicamycin inhibits glycosylation and multiplication of Sindbis and vesicular stomatitus viruses. J. Virol. **21**:375–385.
30. **Lee, J. C., I. Horak, and J. C. Ihle.** 1981. Mechanisms in T cell leukemogenesis. II. T cell responses of preleukemic Balb/c mice to Moloney leukemia virus antigens. J. Immunol. **126**:715–722.
31. **Linemeyer, D. L., J. G. Menke, S. K. Ruscetti, L. H. Evans, and E. M. Scolnick.** 1982. Envelope gene sequences which encode the gp52 protein of spleen focus-forming virus are required for the induction of erythroid cell proliferation. J. Virol. **43**:223–233.
32. **Linemeyer, D. L., S. K. Ruscetti, J. G. Menke, and E. M. Scolnick.** 1980. Recovery of biologically active spleen focus-forming virus from molecularly cloned spleen focus-forming virus-pBR322 circular DNA by cotransfection with infectious type C retroviral DNA. J. Virol. **35**:710–721.
33. **Linemeyer, D. L., S. K. Ruscetti, E. M. Scolnick, L. H. Evans, and D. H. Duesberg.** 1981. Biological activity of the spleen focus-forming virus is encoded by a molecularly cloned subgenomic fragment of spleen focus-forming virus DNA. Proc. Natl. Acad. Sci. U.S.A. **78**:1401–1405.
34. **Machida, C., and D. Kabat.** 1982. Role of partial proteolysis in processing murine leukemia virus membrane envelope glycoproteins to the cell surface. A viral mutant with uncleaved glycoprotein. J. Biol. Chem. **257**:14018–14022.
35. **Mager, D., T. W. Mak, and A. Bernstein.** 1980. Friend leukemia virus-transformed cells, unlike normal stem cells, form spleen colonies in S1/S1$^d$ mice. Nature (London) **288**:592–594.
36. **McGrath, M. S., and I. C. Weissman.** 1979. AKR leukemogenesis: identification and biological significance of thymic lymphoma receptors for AKR retroviruses. Cell **17**:65–75.
37. **Metcalf, D., J. Furth, and R. Buffett.** 1959. Pathogenesis of mouse leukemia caused by Friend virus. Cancer Res. **19**:52–58.
38. **Murray, M., and D. Kabat.** 1979. Genetic and sialylation sources of heterogeneity of the murine leukemia virus membrane envelope glycoproteins gp69/71. J. Biol. Chem. **254**:1340–1348.
39. **O'Farell, P. H., E. Kutter, and M. Nakanishi.** 1980. A restriction map of the bacteriophage-T4 genome. Mol. Gen. Genet. **179**:421–435.
40. **Pinter, A., W. J. Honnen, J.-S. Tung, P. V. O'Donnell, and U. Hammerling.** 1982. Structural domains of endogeneous murine leukemia virus gp70s containing specific antigenic determinants. Virology **116**:499–516.
41. **Polonoff, E., C. Machida, and D. Kabat.** 1982. Glycosylation and intracellular transport of membrane glycoproteins encoded by murine leukemia viruses. Inhibition by amino acid analogues and by tunicamycin. J. Biol. Chem. **257**:14023–14028.
42. **Reddy, E. P., C. Y. Dunn, and S. A. Aaronson.** 1980. Different lymphoid cell targets for transformation by replication-competent Moloney and Rauscher mouse leukemia viruses. Cell **19**:663–669.
43. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
44. **Rowe, W. P., and I. Brodsky.** 1959. A graded response assay for the Friend leukemia virus. J. Natl. Cancer Inst. **23**:1239–1248.
45. **Ruscetti, S. K., D. Linemeyer, J. Feild, D. Troxler, and E. M. Scolnick.** 1979. Characterization of protein found in cells infected with the spleen focus-forming virus that shares immunological cross-reactivity with the gp70 found in mink cell focus-inducing virus particles. J. Virol. **30**:787–798.
46. **Ruta, M., R. Bestwick, C. Machida, and D. Kabat.** 1982. Loss of leukemogenicity caused by mutations in the membrane glycoprotein structural gene of Friend spleen focus-forming virus. Proc. Natl. Acad. Sci. U.S.A. **80**:4704–4708.
47. **Ruta, M., S. Clarke, B. Boswell, and D. Kabat.** 1982. Heterogeneous metabolism and subcellular localization of a potentially leukemogenic membrane glycoprotein encoded by Friend erythroleukemia virus. Isolation of viral and cellular processing mutants. J. Biol. Chem. **257**:126–134.
48. **Ruta, M., and D. Kabat.** 1980. Plasma membrane glycoproteins

encoded by cloned Rauscher and Friend spleen focus-forming virus. J. Virol. **35**:844–853.
49. **Ruta, M., M. J. Murray, M. C. Webb, and D. Kabat.** 1979. A murine leukemia virus mutant with a temperature-sensitive defect in membrane glycoprotein synthesis. Cell **16**:77–88.
50. **Sharp, P. A., P. H. Gallimore, and S. J. Flint.** 1974. Mapping of adenovirus 2 RNA sequences in lytically infected cells and transformed cell lines. Cold Spring Harbor Symp. Quant. Biol. **39**:457–474.
51. **Shields, A., O. N. Witte, E. Rothenberg, and D. Baltimore.** 1978. High frequency of aberrant expression of Moloney murine leukemia virus in clonal infections. Cell **14**:601–609.
52. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol **93**:503–517.
53. **Takatsuki, A., K. Kohno, and G. Tamura.** 1975. Inhibition of biosynthesis of polyisoprenol sugars in chick embryo microsomes by tunicamycin. Agric. Biol. Chem. **39**:2089–2091.
54. **Teich, N., J. Wyke, T. Mak, A. Bernstein, and W. Hardy.** 1982. Pathogenesis of retrovirus-induced disease, p. 785–998. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, molecular biology of tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
55. **Thomas, C. Y., and J. M. Coffin.** 1982. Genetic alterations of RNA leukemia viruses associated with the development of spontaneous thymic leukemia in AKR/J mice. J. Virol. **43**:416–426.
56. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyoxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.
57. **Wendling, F., F. Morean-Gachelin, and P. Tamborin.** 1981. Emergence of tumorigenic cells during the course of Friend disease. Proc. Natl. Acad. Sci. U.S.A. **78**:3614–3618.
58. **Wolff, L., R. Koller, and S. Ruscettti.** 1982. Monoclonal antibody to spleen-forming virus-encoded gp52 provides a probe for the amino-terminal region of retroviral envelope proteins that confers dual tropism and xenotropism. J. Virol. **43**:472–481.
59. **Zielinski, C. C., S. D. Waksal, L. D. Tempelis, R. H. Khiroya, and R. S. Schwartz.** 1980. Surface phenotypes in T-cell leukemia are determined by oncogenic retroviruses. Nature (London) **288**:489–491.