Identification of Genetic Determinants Responsible for the Rapid Immunosuppressive Activity and the Low Leukemogenic Potential of a Variant of Friend Leukemia Virus, FIS-2

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An immunosuppressive variant of Friend murine leukemia virus (F-MuLV), FIS-2, induces suppression of the primary antibody response against sheep erythrocytes (SRBC) in adult NMRI mice more efficiently than the prototype F-MuLV clone 57 (cl.57). It is, however, less potent than F-MuLV cl.57 in inducing erythroleukemia upon inoculation into newborn NMRI mice. Nucleotide sequence analysis shows a high degree of homology between the two viruses. Single point mutations are scattered over both the gag and env encoding regions. The most notable mutations are the deletion of one direct repeat and a few single point mutations occurring in the binding sites for cellular transcriptional factors in the FIS-2 long terminal repeat region (LTR). To define the genetic determinants responsible for the pathogenic properties of FIS-2, we constructed six chimeras between FIS-2 and F-MuLV cl.57. Adult mice were infected with the chimeras, and their primary antibody responses against SRBC were investigated. The results showed that the fragment encompassing the FIS-2 env encoding region SU is responsible for the increased immunosuppressive activity in adult mice. A leukemogenic assay was also performed by infecting newborn mice with the chimeras. Consistent with the previous studies, it showed that the deletion of one direct repeat in the FIS-2 LTR is responsible for the long latent period of erythroleukemia induced by FIS-2 in newborn-inoculated mice. However, studies of cell type-specific transcriptional activities of FIS-2 and F-MuLV cl.57 LTRs using LTR-chloramphenicol acetyltransferase constructs showed that the deletion of one direct repeat does not reduce the transcriptional activity of the FIS-2 LTR. The activity is either comparable to or higher than the transcriptional activity of the F-MuLV cl.57 LTR in the different cell lines that we used, even in an erythroleukemia cell line. It seems that the high transcriptional strength of the FIS-2 LTR is not sufficient to give FIS-2 a high leukemogenic effect. This suggests is inconsistent with the previous suggestion that the transcriptional strength of an LTR in a given cell type is correlated with the leukemogenic potential in the corresponding tissue. In other words, these data indicate that the direct repeats in the F-MuLV LTR may play other roles besides transcriptional enhancer in the leukemogenesis of F-MuLV.

Impairment of the immune system associated with retrovirus infection occurs early in mice infected with the Friend leukemia virus complex (FV) (for reviews, see references 1, 16, and 29). This complex consists of two viral components: a replication-competent helper Friend murine leukemia virus (F-MuLV) and a replication-defective spleen focus-forming virus (30). It induces rapid immune suppression and erythroleukemia of a multistage nature in immunocompetent adult mice of susceptible strains. The symptoms of a general immune suppression which are immediately associated with FV infection in some mouse strains have been considered very similar to those observed in AIDS patients (22, 29). In both cases, the infected host is able to produce virus-specific antibodies, but without efficient clearance of viruses and virus-infected cells (11, 24, 31). Both the humoral antibody response and the cell-mediated immune response against other antigens are reduced (29). Early experiments showed that F-MuLV alone is far less efficient in inducing immunosuppression than FV, although F-MuLV may constitute over 90% of the virus in an FV mixture (29).

Genetic studies showed that the genes located in the H-2 region of the mouse major histocompatibility complex (MHC) control susceptibility to FV-induced immunosuppression (16, 22). Also, the retrovirus transmembrane protein (TM) itself functions as an immunosuppressor because of a conserved immunosuppressive peptide of 17 amino acids (CKS-17) (15, 28). However, the role of the individual viral components of FV in the process of induction of immunosuppression is not clear.

We previously described a variant of F-MuLV clone 57 (cl.57), FIS-2 (9). FIS-2 is much less leukemogenic than F-MuLV cl.57 in newborn-inoculated mice. However, it induces suppression of the primary antibody response more rapidly and efficiently in adult mice than F-MuLV. This immunosuppressive property is comparable to that of FV. Nucleotide sequence analysis showed that FIS-2 and F-MuLV have about 95% homology. Single point mutations are scattered over the whole FIS-2 genome. The noticeable mutations are those located in the long terminal repeat region (LTR) and in the encoding regions of gag and env. However, no mutation occurs in the TM protein, except for an extra amino acid at the carboxyl terminus of the protein. Therefore, this closely related variant of F-
MuLV, FIS-2, provides an opportunity to study the role of a viral element(s) other than the TM protein in retroviral induction of immunosuppression.

In the present studies, we attempted to map the genetic determinant(s) of the pathogenicity of FIS-2, including its property of rapid suppression of the primary antibody response and its leukemogenicity. To do so, we generated a series of chimeric viruses between FIS-2 and F-MuLV c.57. While the leukemogenic potential of each chimera was studied in newborn mice, the immunosuppressive properties of the chimeras were investigated by measuring the primary antibody response against sheep erythrocytes (SRBC) in adult mice. Since several mutations, including the deletion of one direct repeat and a few point mutations, occur in the FIS-2 LTR, we also studied the transcriptional activity of the FIS-2 LTR by using LTR-chloramphenicol acetyltransferase (CAT) constructs and compared it with the transcriptional activities of the F-MuLV LTR and of the F-MuLV LTR with a deletion in one direct repeat in different cell lines. The studies showed the following results.

(i) A region encoding the envelope surface protein of FIS-2 is responsible for the rapid suppression of the primary antibody response exerted by FIS-2.

(ii) The low leukemogenic potential of FIS-2 in newborn-inoculated mice is determined by the FIS-2 LTR, consistent with previous studies (20, 27).

(iii) The transcriptional activity of the FIS-2 LTR is either comparable to or higher than that of the F-MuLV LTR in the different cell lines that we used. This result indicates that the high transcriptional activity of the FIS-2 LTR alone is not sufficient to give FIS-2 a high leukemogenic effect.

MATERIALS AND METHODS

Construction of recombinant viruses. Routine recombinant DNA procedures, such as restriction enzyme digestion, treatment with the modifying enzymes T4 DNA ligase and alkaline phosphatase, purification of DNA fragments from agarose gels, plasmid preparation, and hybridization analysis, were performed as specified by the product manufacturers or by standard methods (25).

Chimeric viruses of FIS-2 and prototype F-MuLV c.57 were constructed. The plasmids used for constructing chimeric viruses are shown in Fig. 1. Plasmid 2-lalc contains the molecularly cloned F-MuLV c.57 genome (17). Plasmid pBR-proFIS-2, which contains the molecularly cloned FIS-2 genome, and plasmid pBR-FIS2, which contains the FIS-2 LTR, gene, and part of the pol gene, were described previously (9). Plasmid pBR-FMuLV was generated by self-ligation of the large EcoRI fragment which contained the F-MuLV LTR, gag, and part of the pol gene and which was derived from plasmid 2-lalc. For construction of plasmid pBR-FIS2-Fmu, the Clal-Kpfl fragment of F-MuLV in plasmid pBR-FMuLV was replaced with the analogous fragment of FIS-2. Similarly, plasmid pBR-FMuLV-FIS2g was generated by substitution of the Clal-Kpfl fragment of F-2 in plasmid pBR-FIS2-FBMuLV with the analogous fragment of F-MuLV.

Plasmids pRE1 and pRE2 were both constructed as a nonpermuted form of proviral DNA inserted into vector pBR322. Plasmid pRE1 was constructed by insertion of a large EcoRI fragment which contained the F-MuLV genome and which was derived from plasmid 2-lalc into the EcoRI site of plasmid pBR-FIS2-gag. Similarity, plasmid pRE2 was constructed by exchanging the Clal-EcoRI fragment at the 5' terminus of the FIS-2 genome in plasmid pBR-FIS2-gag with the analogous fragment of F-MuLV derived from plasmid pBR-FMuLV-gag.

To construct plasmids pRE3 to pRE6, permitted viral DNAs were cloned into vector pBR322. To generate plasmid pRE3, three fragments, the large Scal-EcoRI fragment of pBR322, the EcoRI-ClaI fragment of plasmid 2-lalc containing part of the pol gene and the env gene, and the Clal-Sacl fragment of pBR-FIS2-Fmu, were ligated, purified and ligated together. Plasmids pRE4 and pRE5 were generated by a similar approach. pRE4 was generated by ligation of three fragments, the Scal-EcoRI fragment of pBR322, the analogous EcoRI-ClaI fragment of plasmid pBR-FIS2-gag, and the analogous Clal-Sacl fragment of plasmid pBR-FMuLV-FIS2g. pRE5 was generated by ligation of another combination of three fragments, the Scal-EcoRI fragment of pBR322, the same EcoRI-ClaI fragment of plasmid 2-lalc, and the Clal-Sacl fragment of plasmid pBR-FIS2-gag. For generating pRE6, three fragments, the Scal-ClaI fragment of plasmid pBR-FIS2-gag, the small MscI-ClaI fragment of plasmid pBR-FMuLV-FIS2g, and the small MscI-ClaI fragment of plasmid pBR-proFIS-2 containing FIS-2 env, were ligated.

The structures of all chimeric constructs were validated by digestion with restriction enzymes whose cleavage sites are unique to FIS-2 or to F-MuLV c.57. As all viral genomes were completely assembled, the viral DNA was excised from the plasmids with the appropriate restriction enzymes and ligated to form closed circular DNA or concatamers. NIH 3T3 cells were then transfected with these DNAs. The virus particles produced by transfected NIH 3T3 cells will have the expected proviral structure, as shown in Fig. 1B.

Cell cultures. All the cell lines and media used in this study contained 2 mM L-glutamine and 0.05 mg of gentamicin per ml in addition to 10% newborn calf serum or fetal calf serum. Mouse NIH 3T3 fibroblasts were maintained in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum. L929 is a L-cell line derived from radiation-induced ascitic lymphoma in a C57L mouse (21). EL4-L-2 is a L-cell line derived from a C57BL/6 mouse (12). P88D8 is a monocyte-macrophage cell line originally isolated from a methylcholanthrene-induced lymphoid neoplasm in a DBA/2 mouse (18). CH1 is a B-cell line established from B-cell lymphomas induced by adoptive transfer of syngeneic immune splenocytes in a B10.2H-2-P-4, Win(2-4) mouse (19). SL9 is a Friend erythroleukemia cell line kindly provided by Finn Skou Pedersen. The L691-6 and SL9 cell lines were grown in RPMI 1640 medium supplemented with 10% newborn calf serum. The EL4-L-2 cell line was maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The CH-1 cell line was grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.05 mM 2-mercaptoethanol. The P88D8 cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum.

Transfection of NIH 3T3 cells with DNA clones of infectious virus, virion preparation, and determination of viral titer. NIH 3T3 cells were transfected by the calcium phosphate precipitation method (14). The production of infectious virus was confirmed by the presence of reverse transcriptase activity in cell-free culture supernatants.

For virion preparation, supernatants collected from transfected cells were passed through a 0.45-μm-pore-size filter to remove cellular debris. The filtrates were then centrifuged and then frozen at −80°C. The titers of the viral stocks were assessed by endpoint dilution. Serial 10-fold dilutions were made by use of culture medium containing 8 μg of Polybrene per ml prior to inoculation of NIH 3T3 cells in 24-well plates (2 × 104 cells/well). Four wells for each dilution were inoculated, and cells were passaged three times. The wells with infected cells were identified either by a reverse transcriptase assay or by indirect immunofluorescence. The viral titer range was between 10%/ml and 10%/ml.

The genomic structures of the chimeric viruses were confirmed by Southern blot analysis of Hirt extracts prepared from NIH 3T3 cells infected with the chimeric viral stocks.

Indirect membrane immunofluorescence detection. Monolayers were washed twice with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and then were incubated on ice for 2 h with 250 μl of cell-free supernatants from two hybridoma cell cultures containing monoclonal antibodies 48 and 34, recognizing F-MuLV envelope surface protein gp70 and gag-encoded MA protein, respectively (5). The wells were washed twice, incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (IgG) and IgM antibodies for 1 h, and washed. Infected cells were recognized by their fluorescence.

Preparation of single-cell suspensions of spleenocytes and plaque-forming cell (PFC) assay for primary antibody response. Virus-infected and uninfected control mice were both inoculated intravenously with 108 washed SRBC (State Institute of Public Health, Oslo, Norway). Four days after immunization, spleens were removed into 5 ml of PBS and then frozen at −80°C. The titers of the viral stocks were assessed by endpoint dilution. Serial 10-fold dilutions were made by use of culture medium containing 8 μg of Polybrene per ml prior to inoculation of NIH 3T3 cells in 24-well plates (2 × 104 cells/well). Four wells for each dilution were inoculated, and cells were passaged three times. The wells with infected cells were identified either by a reverse transcriptase assay or by indirect immunofluorescence. The viral titer range was between 10%/ml and 10%/ml.

The number of B cells producing antibodies to SRBC per 1 × 105 or 2 × 105 spleenocytes was determined by a slide monolayer technique (8). Briefly, 1 × 105 or 2 × 105 spleenocytes were mixed with 50 μl of 25% sheep blood and 25 μl of guinea pig serum as a source of complement. The mixture was transferred to a Cunningham slide and incubated at 37°C for 2 h. Plaques were counted by using a Nikon phase-contrast microscope.

Construction of LTR-CAT plasmids. Plasmid pSV2cat (13) was used to construct plasmid pFIS-2LTRCAT by replacement of the Ndel-HindIII fragment containing the simian virus 40 early promoter region with a ClaI-Msel fragment derived from plasmid pBR-FIS2-gag containing the entire FIS-2 LTR. The Ndel site of pSV2cat and the Msel site of the FIS-2 LTR fragment were modified to ClaI and HindIII sites, respectively (see Fig. 4). Plasmid pFMuLVLTRCAT was generated by exchanging the ClaI-Kpfl fragment in pFIS-2LTRCAT with a corresponding fragment derived from plasmid pBR-FMuLV-gag. Plasmid pFMuLVLTRCAT was generated by digestion of pFMuLVLTRCAT with EcoRV and then reassociation of the large fragment.

Transfection of various cell lines with LTR-CAT plasmids and assay of CAT enzyme activity. The cells infected with recombinant viruses, CH1, SL9, NIH 3T3, and P88D8, were transfected by the DEAE-dextran method (26).

For each transfection experiment, different plasmids were transfected into duplicate cell cultures. Transfection experiments were repeated two or three times for each cell line. Cell extracts were prepared by the LTR-CAT assay as described previously (26). The protein content of the cell extracts was deter-
mined by use of the Bio-Rad bicinchoninic acid protein assay system with BSA as a standard.

CAT enzyme activity was determined by previously described methods (26). Instead of liquid scintillation counting, a PhosphorImager was used for analyzing the radioactivity of unacetylated and acetylated chloramphenicol.

Preparation of a digoxigenin-labeled PCR probe and in situ RNA hybridization. The primers for PCR analysis were designed by using the program Oligo 4.1 and were synthesized on an Applied Biosystems 381A synthesizer. Both primers correspond to a sequence in the env encoding region. The forward primer was 5′-GCAAAGACAATAAGTGG-3′ (nucleotide positions 6408 to 6425), and the reverse primer was 5′-TAGTAACCTGTCTCCC-3′ (nucleotide positions 6735 to 6749). PCR was performed by a modification of a method described by Borg et al. (2). Briefly, PCR mixtures consisted of 50 mM KCl, 20 mM Tris-HCl (pH 8.83), 1.5 mM MgCl2, 15 pmol of each primer, 5 μl of 100-fold-diluted DNA sample, 1 mM each deoxynucleoside triphosphate, and 0.3 mM digoxigenin-dUTP in a total volume of 50 μl. The mixture was covered with 100 μl of mineral oil (Sigma) and heated at 95°C for 5 min. One unit of Taq polymerase was added to the mixture (hot start). The program for amplification was 35 cycles for 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C.

The protocol for in situ RNA hybridization was based on several procedures described previously (32). Cryostat sections of fresh-frozen spleen with a thickness of 12.5 μm were used for in situ hybridization. After the slides were fixed with PBS containing 1% glutaraldehyde and washed, permeabilization was done with TE buffer (0.1 M Tris-HCl, 50 mM EDTA [pH 8.0]) containing 1 μg of proteinase K per ml for 10 min at 37°C. This treatment was followed by two rinses with PBS, re-fixation, an H2O rinse, incubation with 0.1 M triethanolamine (pH 8.0) for 1 min, incubation with 0.25% acetic anhydride–0.1 M triethanolamine for 5 min, two rinses with H2O, dehydration with 50, 70, 95, and 100% ethanol, and air drying.

Slides of tissue sections were covered with a hybridization solution consisting of 1× hybridization buffer (0.3 M NaCl, 20 mM Tris-HCl [pH 7.2], 5 mM EDTA [pH 8.0]), 1× Denhardt’s solution, 50% formamide, 10% dextran sulfate, 0.25 μg of yeast tRNA per μl, and 0.25 ng of denatured digoxigenin-labeled PCR probe per μl. Hybridization was carried out at 50°C overnight. Slides were then washed with SSC solutions (2× SSC at 50°C for 20 min, 0.2× SSC at 50°C for 20 min, 2× SSC at room temperature for 5 min) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and finally rinsed with H2O.

Probe detection was performed with alkaline phosphatase-conjugated antidigoxigenin antibody (anti-DIG-AP) (Boehringer Mannheim Biochemicals). Before reactions were detected, slides were washed once with detection buffer (1%
RESULTS

Structure of chimeric viruses of FIS-2 and F-MuLV cl.57. The proviral forms of six recombinant viruses of FIS-2 and F-MuLV cl.57 are illustrated in Fig. 1B. Among them, RE1, RE3, and RE5 were significantly different from FIS-2 (P < 0.05). Recombinant viruses RE2, RE4, and RE6, however, suppressed the primary anti-SRBC response in NMRI mice to the same extent as did FIS-2 (P > 0.05). While the anti-SRBC response in the majority of the RE4-infected mice was suppressed to a very low level, infection with both RE2 and RE6 abrogated the response completely. Figure 2 also shows that the relative immunosuppressive activity in individual mice infected with RE1, RE3, and RE5 varied greatly, as did that in mice infected with F-MuLV cl.57. It seemed that the Ndel-ClaI fragment of FIS-2 contained a main, dominant determinant contributing to the efficient immunosuppressive activity of FIS-2.

The Ndel-ClaI fragment of FIS-2 contains a region of 374 nucleotides upstream of the env encoding region. The splice acceptor site is located in this region. Nucleotide sequence analysis showed high homology between FIS-2 and F-MuLV cl.57 in this region. Only two amino acids were mutated in the integrate encoding region as a result of three point mutations (T to C at site 5755, G to T at site 5767, and A to G at site 5769; pol gene was switched between FIS-2 and F-MuLV cl.57. RE3 and RE4 were generated by exchanging the ClaI-KpnI fragment containing the entire U3 region and part of the R region. To study the pathogenic effect contributed by the FIS-2 env gene, RE6 received the Ndel-ClaI fragment of FIS-2 containing almost the whole env gene of FIS-2 in the F-MuLV cl.57 background.

The fragment encompassing FIS-2 env is responsible for the FIS-2-induced rapid suppression of the primary antibody response in adult NMRI mice. Previous studies showed that infection of adult NMRI mice with FIS-2 more efficiently causes a failure in the primary antibody response against SRBC than does F-MuLV cl.57. To understand the molecular mechanisms of this pathogenicity of FIS-2, we infected adult NMRI mice with recombinant viruses of FIS-2 and F-MuLV cl.57. Both infected and uninfected control mice were immunized with SRBC 10 days postinfection. Their primary antibody responses against SRBC were studied by performing the PFC assay 4 days after immunization. The number of plaques per 1 × 10^6 or 2 × 10^6 splenocytes, formed by B cells producing anti-SRBC IgM, was counted. Due to variations between different experiments, the PFC values for individual mice infected with either parental or recombinant virus were normalized to the PFC values for the control group of uninfected mice in the same experiment (Fig. 2). The actual PFC values for the control groups and the number of mice used in each experiment are shown in Table 1. Expressing the degree of immunosuppression as the percentage of mice with relative PFC values below 10% showed that recombinant viruses RE1, RE3, and RE5 were significantly different from FIS-2 (P < 0.002). Recombinant viruses RE2, RE4, and RE6, however, suppressed the primary anti-SRBC response in NMRI mice to the same extent as did FIS-2 (P > 0.05). While the anti-SRBC response in the majority of the RE4-infected mice was suppressed to a very low level, infection with both RE2 and RE6 abrogated the response completely. Figure 2 also shows that the relative immunosuppressive activity in individual mice infected with RE1, RE3, and RE5 varied greatly, as did that in mice infected with F-MuLV cl.57. It seemed that the Ndel-ClaI fragment of FIS-2 contained a main, dominant determinant contributing to the efficient immunosuppressive activity of FIS-2.

### Table 1. Actual PFC values for control groups and number of mice used in each experiment

<table>
<thead>
<tr>
<th>Virus</th>
<th>Actual mean no. of PFCs for control mice ± SE (no. of control mice/no. of infected mice) in expt:</th>
<th>% of infected mice with relative PFC values below 10%</th>
<th>P &lt; a</th>
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<tr>
<td></td>
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<tr>
<td>RE1</td>
<td>82.4 ± 27.1 (5/10)</td>
<td>10.0</td>
<td>0.002</td>
</tr>
<tr>
<td>RE2</td>
<td>155.8 ± 88.1 (6/7)</td>
<td>91.7</td>
<td>0.35</td>
</tr>
<tr>
<td>RE3</td>
<td>155.8 ± 88.1 (6/6)</td>
<td>91.7</td>
<td>0.35</td>
</tr>
<tr>
<td>RE4</td>
<td>155.8 ± 88.1 (6/6)</td>
<td>91.7</td>
<td>0.35</td>
</tr>
<tr>
<td>RE5</td>
<td>155.8 ± 88.1 (6/6)</td>
<td>91.7</td>
<td>0.35</td>
</tr>
<tr>
<td>RE6</td>
<td>536.0 ± 146.7 (5/5)</td>
<td>100.0</td>
<td>0.31</td>
</tr>
<tr>
<td>FIS-2</td>
<td>216.0 ± 82.0 (5/5)</td>
<td>100.0</td>
<td>1.00</td>
</tr>
<tr>
<td>F-MuLV</td>
<td>216.0 ± 82.0 (5/5)</td>
<td>100.0</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Between mean PFC values for mice infected with FIS-2 versus other viruses (determined with the Student t test).
No mutations occurred at or around the splice acceptor site, which is important for the production of spliced and unspliced mRNAs at a proper ratio (23). Northern blot analysis of viral RNA in NIH 3T3 cells infected with either FIS-2 or F-MuLV cl.57 also demonstrated the proper ratio of spliced and unspliced mRNAs (data not shown). Since both FIS-2 and F-MuLV cl.57 established infection efficiently in cell cultures, it seemed very unlikely that the two changed amino acids affected integrase functionally. Therefore, it was concluded that the part of the FIS-2 \textit{env} encoding region in the NdeI-ClaI fragment was the major determinant for the immunosuppressive pathogenicity of FIS-2.

There is no direct correlation between the amount of virus expression in the spleen and immunosuppressive activity. To test if virus expression was higher in spleens from mice with complete suppression of the primary antibody response against SRBC than in spleens from mice with only partial suppression of the anti-SRBC response, in situ RNA hybridization was performed with tissue sections of spleens from the two groups. An \textit{env}--specific PCR probe was used to detect total viral RNA, including unspliced and spliced mRNAs. In each hybridization experiment, tissue sections prepared from an infected spleen were used as a positive control. Various levels of virus expression were observed in spleens from both groups. Figure 3 shows four representative specimens. Spleens from two totally immunosuppressed mice infected with RE2 showed different levels of expression (Fig. 3A and B). Similarly, the primary anti-SRBC response was less depressed in a mouse with a high level of expression of RE1 in the spleen than in a mouse with a low level of expression (Fig. 3C and D).

Furthermore, histological examination revealed no obvious morphological changes in spleens from mice infected for 2 weeks. Therefore, the failure of a primary antibody response against SRBC was not due to anatomical derangement but rather was due to the microenvironmental changes induced in the immune system by the virus infection.

The low leukemogenic potential of FIS-2 is determined by the FIS-2 LTR but is not correlated with the high transcriptional activity of the FIS-2 LTR. Previous studies showed that FIS-2 induced erythroleukemia with a much longer latent period than F-MuLV cl.57 (9). Studies of chimeric viruses in newborn mice showed that the FIS-2 LTR was responsible for the low leukemogenic potential of FIS-2 (data not shown). The chimera RE3, which was generated by substituting the F-MuLV LTR for the FIS-2 LTR, induced erythroleukemia with a latency period similar to that seen with FIS-2 (141 days for RE3 and 135 days for FIS-2). The leukemogenicity of FIS-2 increased as the FIS-2 LTR was replaced with the F-MuLV LTR (RE4). RE4 induced disease with a latency period of 33 days, even shorter than that seen with F-MuLV (46 days). That a deletion in the tandem repeat of the F-MuLV LTR attenuated the leukemogenesis of F-MuLV was shown previously by either deletion analysis (20) or studies of a naturally occurring strain of F-MuLV (27). Although a different region of the tandem repeat was deleted in the FIS-2 LTR compared to the
deletions studied previously (9, 20, 27), our results emphasize the fact that the presence of both direct repeats in the LTR is necessary for the high leukemogenic potential of F-MuLV.

It has been suggested that there is a correlation between the transcriptional strength of the LTR from murine leukemia virus in a given cell type and the leukemogenic potential of the virus in the corresponding tissue (3, 4, 26). Therefore, we were interested in studying the transcriptional activity of the LTRs from FIS-2 and F-MuLV in different lymphoid cell lines. We constructed plasmids pFIS-2LTRCAT and pFMuLVLTRCAT, in which the expression of the CAT gene is directed by the FIS-2 LTR and the F-MuLV LTR, respectively (Fig. 4). Because the FIS-2 LTR contains only one direct repeat which is highly homologous to the first direct repeat in the tandem repeat from F-MuLV LTR with a few point mutations, we were also interested in comparing the transcriptional activity of the FIS-2 LTR with that of an F-MuLV LTR which had one direct repeat deleted. For this purpose, we constructed plasmid pΔFMuLVLTRCAT, in which one direct repeat is deleted in the F-MuLV LTR.

Several lymphoid cell lines of different types, including T, B, monocyte-macrophage, and erythroleukemia cells, were chosen for DNA transfection with the three plasmids. Expression of the CAT gene was monitored by measuring CAT enzyme activity in cell lysates prepared after transfection of cells for 48 h. For each transfection, the CAT activity of cell lysates prepared from cell cultures transfected with plasmid pFMuLV LTRCAT was arbitrarily set at 100%. As shown in Table 2, although the FIS-2 LTR contains only one direct repeat in its enhancer region, in all the cell lines studied it had transcriptional activity either higher than (in cell lines L691-6, CH-1, and p388D1) or similar to (in cell lines EL4.IL-2 and SL9) that of the F-MuLV LTR, containing two perfect direct repeats.

The results also showed that the deletion of one direct repeat in the F-MuLV LTR affected transcriptional activity differently in different cell lines. In some cell lines, such as EL4.IL-2, P388D1, CH-1, and SL9, the loss of one direct repeat in the F-MuLV LTR caused a decrease in transcriptional activity. In particular, in cell line EL4.IL-2, the effect was so strong that the deletion of one direct repeat abrogated almost all the LTR activity. However, no significant effects were observed in cell line L691-6. These results indicated that the few single point mutations in the FIS-2 LTR had compensated for the loss of one direct repeat and even had made it become a more efficient and stronger transcriptional unit in some cell types. The fact that the FIS-2 LTR is the determinant for the low leukemogenic effect of FIS-2 despite its high transcriptional activity, even in an erythroleukemia cell line, was inconsistent with previous suggestions. Therefore, it was suggested that the role of the FIS-2 LTR in attenuating the leukemogenesis of FIS-2 is not necessarily played via down regulation of transcriptional activity.

**DISCUSSION**

Suppression of primary antibody responses induced by retroviruses is a complex process involving many different cell types. The mechanism by which retroviruses down regulate the immune response is not yet fully understood. One hypothesis is that the viral LTRs, which are required for viral replication, also contain sequences that can down regulate the expression of certain genes. The results of this study suggest that the FIS-2 LTR, which is the determinant for the low leukemogenic effect of FIS-2, contains sequences that can down regulate the expression of certain genes.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Relative CAT activity with:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>pFMuLVLTRCAT</td>
</tr>
<tr>
<td>EL4.IL-2</td>
<td>T</td>
<td>100 (3)</td>
</tr>
<tr>
<td>L691-6</td>
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<td>CH-1</td>
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<td>100 (2)</td>
</tr>
<tr>
<td>P388D1</td>
<td>Monocyte-macrophage</td>
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</tr>
<tr>
<td>SL9</td>
<td>Erythroleukemia</td>
<td>100 (2)</td>
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* For each experiment, three plasmids were transfected into duplicate cell cultures. All cultures were transfected by the DEAE-dextran method. CAT activities were analyzed by the computer program MD Image Quant for a PhosphorImager and were normalized relative to the value for pFMuLVLTRCAT from the same transfection experiment.

* Numbers in parentheses indicate the numbers of transfection experiments.

* Reported as mean ± SD (P < 0.05). P values represent the levels of significance of CAT activities for pFMuLVLTRCAT versus those of the other CAT plasmids.
types in the immune system. Many studies have shown that the retroviral TM protein confers general immunosuppressive effects due to a conserved peptide of 17 amino acids (CKS-17) (15, 28). However, the role of other viral factors is unclear. In previous studies (9), we described FIS-2, a closely related variant of F-MuLV cl.57 that has an enhanced suppressive effect on the primary antibody response. The majority of mice infected with FIS-2 had no ability to generate B cells producing primary antibodies to SRBC 2 weeks after infection, whereas a variable response was observed among mice infected with F-MuLV cl.57. Since FIS-2 and F-MuLV cl.57 have almost identical nucleotide sequences encoding the TM protein, the main objective of this study was to determine the viral factors other than the TM protein in FIS-2 that underlie the enhanced immunosuppressive properties of FIS-2. To achieve this, we constructed six different recombinant viruses of FIS-2 and F-MuLV cl.57. Immunocompetent adult mice were inoculated with these viruses, and their primary anti-SRBC responses were investigated.

Studies of the six recombinant viruses of FIS-2 and F-MuLV cl.57 showed large variations in the primary anti-SRBC responses among individual mice infected with RE1, RE3, and RE5. Similar individual variations were also observed for mice infected with F-MuLV cl.57. Earlier studies showed that the genotype of the mouse MHC (H-2) affects susceptibility to murine retrovirus-induced suppression of immunological responses, such as the response to SRBC (22). Although our experiments were performed with NMRI mice (an outbred mouse strain), all NMRI mice have the same genotype of the MHC (H-2K(d)) (data sheet about NMRI mice supplied by Bomholdgaard Breeding Research Center). Therefore, variations in the levels of anti-SRBC PFCs in individual infected mice were not due to differences in the genotype of MHC (H-2). Nor were they due to a low susceptibility of NMRI mice to the viruses, since newborn NMRI mice were extremely susceptible to erythroleukemia induction by F-MuLV cl.57 and RE1. They were rather due to other undefined and various genetic factors of the NMRI mouse strain. Interestingly, the varied genetic background of the mice seemed to have no influence or much less influence on the suppression of anti-SRBC responses induced by FIS-2. Our experiments showed that the NdeI-ClaI fragment of FIS-2, which encompasses FIS-2 envelope protein SU, was responsible and necessary for giving FIS-2 enhanced immunosuppressive activity. This result suggests that the suppressive effect of this fragment is overwhelming and thus is dominant over effects of various genetic factors of individual NMRI mice. Although the level of expression of the TM protein was not analyzed directly in this study, it was assumed to be proportional with the level of expression of spliced mRNA, which was proportional with the level of expression of total mRNA. In situ RNA hybridization was used to demonstrate the extent of virus infection and the expression of virus RNA in the spleen. No direct correlation was found between the extent of general virus infection in the spleen and the level of immunosuppression. The primary antibody response in mice infected with RE1 was not reduced, despite an efficient virus infection in the spleen. A possible explanation for this finding is that the suppression of a primary anti-SRBC response by virus occurs via interference with immune cells, not only in the spleen but at other sites of the immune system. The variable extent of suppression of the anti-SRBC response associated with F-MuLV infection may also suggest that the general immunosuppressive action exerted by the TM protein alone is strongly influenced by the genetic background of the mice, unless a dominant factor, such as FIS-2 SU, is present. Since previous experiments were performed with inbred mouse strains, such variations would not have been observed.

In this study, we also made another notable observation. Although the loss of one direct repeat in the FIS-2 LTR caused a long latency for erythroleukemia, it did not cause a significant decrease in its transcriptional activity in all the cell lines studied, even in a Friend erythroleukemia cell line. It seems that a few point mutations have converted the FIS-2 LTR to a highly active transcriptional unit. Because the direct repeat in the LTR region is associated with a transcriptional enhancer, a correlation between the transcriptional strength of the LTR in a given cell lineage and the leukemogenic potential of the virus in the corresponding tissue has been suggested (3, 4, 26). However, our experiments demonstrated that such a correlation does not necessarily exist, since the high transcriptional activity of the FIS-2 LTR in vitro did not result in a high leukemogenic potential of FIS-2. In situ RNA hybridization analysis of spleens from 10-day-old mice infected with F-MuLV cl.57 or FIS-2 as newborns showed that the level of expression of FIS-2 in the spleens was either higher than or at least comparable to the level of expression of F-MuLV cl.57 in the spleens (10). Our data suggest that the high transcriptional activity of an LTR is probably not sufficient to contribute to the corresponding murine leukemia virus a high leukemogenic potential.

The results of this study raise the question of whether there are any additional roles of the two direct repeats in the LTR region involved in the leukemogenicity of F-MuLV, since the role of giving high transcriptional activity is not sufficient. Recently, it was reported that the Moloney murine leukemia virus (MoMLV) LTR encodes a novel transcript, let (LTR-encoded transactivator), which is a product of RNA polymerase III (6, 7). This RNA product can transactivate several genes that belong to the immunoglobulin superfamily, such as class I MHC antigens. It was suggested that the leukemogenicity of MoMLV is associated with transactivation of cellular genes by let (11a). Although there is no direct evidence of the relationship between let and the potential leukemogenicity of MoMLV, nor is it known whether the LTRs of F-MuLV and of FIS-2 encode a transcript similar to let, it might be interesting to investigate such a possibility both in vitro and in vivo.

In conclusion, the present study shows that the SU region in FIS-2 is necessary for FIS-2 to exert efficient suppression of primary anti-SRBC responses in adult NMRI mice. It also shows that although the low leukemogenicity of FIS-2 is related to the deletion of one direct repeat in the U3 region of the LTR, it is not correlated with a loss of transcriptional strength of the FIS-2 LTR. To our knowledge, this is the first evidence that the high transcriptional activity of an LTR in vitro is not correlated with the potential leukemogenicity of the corresponding virus. How a virus such as FIS-2, which induces immunosuppression so rapidly and efficiently in adult mice, displays such low leukemogenic activity in newborn-inoculated mice, despite its high level of expression, is not understood. Therefore, our future studies will be focused on exploring other, alternative roles of the direct repeat in the LTR region in the induction of erythroleukemia by F-MuLV. We will also study the role of the FIS-2 envelope protein in the process of suppression of the primary antibody response. In particular, the immunological and functional changes in virus-infected cells will be defined and analyzed.

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