

# FRIEND VIRUS-SPECIFIC CYTOTOXIC T LYMPHOCYTES RECOGNIZE BOTH *gag* AND *env* GENE-ENCODED SPECIFICITIES

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Antitumor CTL are generated by immunization of *H-2<sup>b</sup>* mice with Friend erythroleukemia virus complex (FV)<sup>1</sup> or with syngeneic FV-induced tumor cells (1–3). These effector cells can recognize and lyse only those cells expressing cell-surface antigens of both FV and a class I MHC molecule of the *H-2<sup>b</sup>* haplotype. Previous studies (2) indicated that only the *H-2D<sup>b</sup>* and not the *H-2K<sup>b</sup>* molecule can participate in the formation of the target structure(s) recognized by these CTL, but the viral molecule(s) remain to be identified. Viral proteins that appear on the surface of infected cells are encoded by the *env* and *gag* genes of each of the two component viruses of FV, helper Friend murine leukemia virus (FMuLV) and replication-defective spleen focus-forming virus (SFFV) (4, 5). These molecules are the FMuLV *env*-gene encoded glycoproteins gp70 and p15(E), and the glycosylated *gag* membrane proteins gP85 and its precursor gP95 (6); present in lesser amounts on the cell surface are the SFFV *env* product gp52 and *gag*-encoded protein p45 (7). Several investigators have used a variety of indirect approaches to identify the MuLV-encoded molecule(s) recognized by CTL. Studies using purified gp70<sup>env</sup> or anti-gp70 antibodies to block CTL recognition of virus-infected target cells have yielded contradictory results (8–10). Experiments with mutant cell lines lacking detectable expression of either gp70<sup>env</sup> or gP85<sup>gag</sup> have implicated these molecules as targets for CTL recognition (11, 12). A more direct approach using a recombinant DNA clone that expressed gp70<sup>env</sup> and p15(E)<sup>env</sup> of Moloney murine leukemia virus upon transfection into target cells demonstrated the existence of a population of virus-specific CTL able to recognize these molecules (13).

We now present direct evidence for the participation of both *env* and *gag* gene-encoded cell-surface proteins in target structure formation. To identify the viral and H-2 antigens recognized by anti-FV CTL, cell lines expressing putative target molecules, either singly or in combination, were constructed by DNA-mediated gene transfer. Since FV is of murine origin, it was advantageous to use cells of a heterologous species (rat) as the parental line for the derivation of these “synthetic” target cells. This approach allowed an evaluation of the relative

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<sup>1</sup> *Abbreviations used in this paper:* F-MCF, Friend mink cell focus-inducing virus; FMuLV, Friend murine leukemia virus; FRE, Fisher rat embryo fibroblast; FV, Friend erythroleukemia virus complex; LTR, long terminal repeat; MLC, mixed lymphocyte culture; SFFV, spleen focus-forming virus.

contribution of each transfected mouse gene to the formation of cell-surface target structures recognized by CTL without interference from endogenous murine proteins and viral sequences. FRE cells, an established line of Fisher rat embryo fibroblasts, were used in this study, as they do not express detectable levels of rat type C viral RNA or structural proteins (14), and are readily infected with virus or transfected with cloned DNA. The results indicate that transfected murine histocompatibility genes can be stably expressed in FRE cells, that their products are recognized by murine allospecific CTL, and that the transfected  $D^b$  molecule can serve as a restricting element for anti-FV CTL generated in BALB.B ( $H-2^b$ ) mice. The data also demonstrate that both FMuLV and SFFV provide viral antigens that are recognized, in association with  $D^b$ , by both anti-FV and anti-FMuLV CTL populations; furthermore, the FMuLV specificities that serve as targets are encoded by both the *env* and *gag* genes. The use of heterologous cell lines for the construction of synthetic target cells thus offers a useful approach for the analysis of the interactions between T cells and their targets.

### Materials and Methods

*Mice.* All mice were bred at the Albert Einstein College of Medicine, Bronx, NY. The inbred strains used included the H-2-congenic strains BALB/cAn ( $H-2^d$ ), BALB.B ( $H-2^b$ ), BALB.G ( $H-2^g$ ), and BALB.5R ( $H-2^{i\beta}$ ). Female mice aged 6–12 wk were used in these studies.

*Cells and Viruses.* Fisher rat embryo (FRE) clone 2 fibroblasts (14) were used as the parental cell line for the construction of target cells. SFFV<sub>p</sub>-FRE are FRE cells nonproductively infected with a biological clone of SFFV derived from the Lilly-Steeves strain of polycythemia-inducing FV (15) by end-point dilution, and EY-10 are FRE cells productively infected with both SFFV<sub>p</sub> and a biological clone of FMuLV<sub>A</sub> derived from an anemia-inducing strain of FV (16). A cell line productively infected with FMuLV<sub>A</sub> only (F<sub>201</sub>NIH) (17) was maintained in parallel as a source of cloned helper virus, which could be used to infect FRE cells. The chemically induced C57BL/6 lymphoma cell line EL4 served as an  $H-2^b$  control. All lines were grown in DME supplemented with 10% FCS, glutamine, and antibiotics.

*Establishment of Cell Lines Expressing H-2 and/or Viral Proteins.* DNA transfections of uninfected or virus-infected FRE cells used a modification of the calcium phosphate precipitation method (18). Plasmids containing the  $D^b$  or the  $K^b$  class I genes (19, 20) were digested with Hind III and Eco RI (New England Biolabs, Beverly, MA), respectively, to release the cloned insert from vector sequences. 15  $\mu$ g of H-2 DNA and 1  $\mu$ g of the dominant selectable marker pSV2neo (21) were coprecipitated (22) in a final volume of 0.5 ml and applied to a culture of  $3 \times 10^5$  cells plated 24 h earlier. After 2 d, cells were split 1:10 onto 60-mm dishes in the presence of 800  $\mu$ g/ml G418 (geneticin sulfate; Gibco Laboratories, Grand Island, NY). Transfection of FRE cells with 1  $\mu$ g of intact pSV2neo yielded stable G418-resistant transformants at a frequency of  $5 \times 10^{-5}$ . After 2 wk, individual colonies of G418-resistant cells were visualized, isolated using cloning cylinders, expanded in tissue culture, and screened for H-2 expression by RIA.

Molecular construction of subgenomic viral expression vectors was performed according to standard techniques (23). The identity of recombinant clones were verified by extensive restriction enzyme analysis. Plasmid DNA was isolated from *Escherichia coli* strain C-600 by the alkaline lysis method, and was purified on two successive cesium chloride gradients. Plasmid 2-lac contains a complete copy of the FMuLV<sub>A</sub> provirus; it was derived from plasmid 2-lal (pFMuLV<sub>57</sub>) (24, 25), which consists of a circularly permuted copy of the viral genome of cell line F<sub>201</sub>NIH molecularly cloned into pBR322. The FMuLV *gag* expression vector, 2-lalc $\Delta$ s, and the viral *env* vector, pSV2Fe/gpt-1, were converted to linear form by restriction enzyme digestion and separately transfected

onto uninfected FRE cells along with  $D^b$  and pSV2neo. G418-resistant colonies were isolated, expanded in vitro, and screened for expression of H-2 and viral gene products.

**Antisera.** H-2 and viral proteins were detected using mouse ascites fluid from the following monoclonal hybridoma cell lines: 28-14-8 (anti- $D^b$ ) (26), EH-144 (anti- $K^b$ ) (unpublished; provided by Dr. T.V. Rajan, Albert Einstein College of Medicine), 55 (anti-FMuLV gp70<sup>env</sup>) (27), and 34 (anti-FMuLV/SFFV p15<sup>gag</sup>) (27, 28). The latter also reacts with the p15 components of gp85 and gp95, the glycosylated cell-surface products of FMuLV *gag*, and of p45, the cell-surface protein encoded by the *gag* gene of the SFFV<sub>P</sub> strain used in these studies. Goat antisera to Rauscher murine leukemia virus gp70<sup>env</sup> and Pr60<sup>gag</sup> antigens were obtained through the Office of Program Resources and Logistics, Viral Oncology Division, National Cancer Institute, Bethesda, MD. All antisera were heat-inactivated at 56°C for 30 min.

**Radioimmunoassay.** RIA were performed in Falcon 96-well flexible assay plates (Becton Dickinson, Oxnard, CA). Log-phase cell cultures were briefly trypsinized, and  $4 \times 10^5$  viable cells were added to triplicate wells in 20  $\mu$ l of RIA buffer (Eagle's MEM supplemented with antibiotics, glutamine, 0.06% sodium azide, and 5% FCS). 20  $\mu$ l of appropriately diluted hybridoma ascites fluid (titrated in separate experiments) were added per well; the contents were mixed and incubated for 60 min at 4°C. The cells were washed three times by vigorously resuspending in 150  $\mu$ l buffer and centrifuging for 5 min at 300 g. The cells were then incubated for 60 min at 4°C with  $2 \times 10^4$  cpm <sup>125</sup>I-protein A (New England Nuclear, Boston, MA) in 50  $\mu$ l/well. After four more washes, cell pellets in individual wells were cut apart and measured for radioactivity in a gamma counter. Values represent the means of total counts per minute bound from three replicate wells. Standard errors were always <5% of mean value and have been omitted from the tables.

**Cell Labeling and Immunoprecipitations.** Subconfluent monolayers were metabolically labeled with 100  $\mu$ Ci/ml of [<sup>3</sup>H]leucine (Amersham Corp., Arlington Heights, IL) in leucine-free medium containing 1% dialyzed FCS. Labeling was carried out for 30 min at 37°C.

Pulse-labeled cells were washed in cold PBS, and then lysed in extraction buffer (0.01 M sodium phosphate buffer, pH 7.6, 0.1 M NaCl, 1% Triton X-100, 0.5% deoxycholate, 1 mM EDTA, and 0.1% SDS). Extracts were spun at 2,000 g for 20 min and precleared overnight by incubation with normal goat serum (5  $\mu$ l/ml) and 50  $\mu$ l/ml of a 10% formalin-fixed solution of *Staphylococcus aureus*. Extracts were clarified by centrifugation at 2,000 g for 20 min. Immunoprecipitations were set up by incubating 1 ml of each extract with 5  $\mu$ l of goat antiserum; after 16 h of incubation at 4°C, 50  $\mu$ l of *S. aureus* solution was added and the incubation was continued for 2 h more. Reaction tubes were then centrifuged at 2,000 g for 20 min. The pellets were washed three times with extraction buffer, and were resuspended and boiled for 1 min in electrophoresis sample buffer (50 mM Tris HCl pH 6.8, 1% SDS, 1% 2-ME, 10% glycerol, and 0.001% bromophenol blue) in preparation for SDS-PAGE.

7% polyacrylamide gels with 3.5% stacking gels were prepared, and electrophoresis was carried out by the Laemmli procedure (29). [<sup>14</sup>C]-labeled molecular weight markers (Bethesda Research Laboratories, Gaithersburg, MD) were run with each gel. Gels were then fixed and fluorographed by the method of Bonner and Laskey (30) and exposed to Kodak XAR-5 film at -70°C.

**Lymphocyte Cultures.** Allogeneic secondary mixed lymphocyte cultures (MLC) were established to generate CTL specific for H-2<sup>b</sup> antigens. Allospecific CTL were generated in BALB/c ( $K^dD^d$ ) mice immunized twice by i.p. inoculation of  $2.5 \times 10^7$  untreated allogeneic spleen cells: BALB.B ( $K^bD^b$ ), anti-H-2<sup>b</sup>; BALB.G ( $K^dD^b$ ), anti- $D^b$ ; and BALB.5R ( $K^bD^d$ ), anti- $K^b$ . Secondary MLC were established by restimulating  $2.5 \times 10^7$  immune BALB/c spleen cells in vitro with  $2.5 \times 10^7$  irradiated (5,000 rad) allogeneic spleen cells (as above) in upright tissue culture flasks containing 20 ml RPMI 1640 medium (Gibco Laboratories) supplemented with antibiotics, glutamine,  $5 \times 10^{-5}$  M 2-ME, and 5% FCS. After 6 d, responder spleen cells were collected, purified on Isopaque/Ficoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradients (31), and assayed for cytolytic activity in a <sup>51</sup>Cr-release cytotoxicity assay.

Virus-specific CTL were generated in BALB.B ( $H-2^b$ ) mice against both components of FV, FMuLV and SFFV, or against FMuLV alone. Spleen cells from BALB.B mice infected 10–14 d earlier by i.v. inoculation of 1 ml EY-10 culture supernatant (FMuLV + SFFV) were glutaraldehyde-fixed (32) to prevent shedding of infectious virus particles. Alternatively, spleen cells from BALB.B mice infected 2–3 mo earlier (as neonates) with 0.2 ml  $F_{201}$ NIH culture supernatant (FMuLV only) i.p. were inactivated by irradiation (5,000 rad).  $2.5 \times 10^7$  treated cells were inoculated i.p. into 6–8-wk-old BALB.B recipients; after 4 wk, a second *in vivo* immunization followed. Immune spleen cells were harvested 2 wk later, and syngeneic mixed lymphocyte-tumor cell cultures (MLTC) were established by restimulating  $2.5 \times 10^7$  responder cells *in vitro* with  $10^6$  irradiated (5,000 rad) FV- or FMuLV-infected BALB.B spleen cells under the culture conditions described above. After 6 d at 37°C, responder spleen cells were harvested, purified, and assayed for cytolytic activity.

<sup>51</sup>Cr-release Cytotoxicity Assay. Cell-mediated cytolytic activity was detected by incubating  $10^4$  <sup>51</sup>Cr-labeled control or FRE-derived target cells and varying numbers of immune lymphocytes. Cells were mixed in a final volume of 0.2 ml MEM supplemented with antibiotics, glutamine, and 10% FCS in triplicate wells in conical-bottom multiwell plates, and incubated at 37°C. After 6 h, the supernatants were harvested and tested for radioactivity in a gamma counter. The percent specific <sup>51</sup>Cr release was calculated for each lymphocyte/target cell ratio as: percent release =  $100 \times \{(\text{experimental release}) - (\text{spontaneous release})\} / [(\text{maximum release}) - (\text{spontaneous release})]$ , where the spontaneous <sup>51</sup>Cr release was detected after incubation of target cells in medium alone and the maximum <sup>51</sup>Cr release was obtained after incubation of target cells in 1 N HCl. The spontaneous <sup>51</sup>Cr release of all FRE-derived targets was <25%, and <15% for EL4. Release values represent the mean specific <sup>51</sup>Cr release from three replicate wells. Standard errors were always <5% of mean value and have been omitted from the tables. All assays were carried out at four to six different E/T ratios. Representative results from one ratio are presented in the tables.

## Results

*H-2 Expression in Transfected FRE Cells.* Uninfected FRE, SFFV-infected FRE, and FRE infected with both SFFV and FMuLV (EY-10 cells) were transfected with either the  $D^b$  or the  $K^b$  class I genes, along with the selectable marker pSV2neo. G418-resistant cell clones were selected and screened for expression of H-2 and viral cell-surface antigens by RIA using specific monoclonal antisera. Cell monolayers were routinely harvested for assay by brief trypsinization (3 min at 37°C). Although trypsin is a nonspecific protease, no significant effect on the level of H-2 or FV cell-surface protein expression has been detected after such treatment (data not shown). Cells resuspended from non-tissue culture-treated bacteriological petri dishes or collected by incubation with 0.02% Versene (Gibco Laboratories) at 37°C for 20 min expressed similar levels of cell-surface antigen.

60–80% of the G418-resistant clones screened expressed detectable levels of H-2 antigen; amounts varied from slightly above background levels to more than twice that of control  $H-2^b$  cell lines (e.g., the chemically induced lymphoma line EL4). Data from representative clones selected to serve as target cell lines appear in Table I. Cell lines B2 and R19 were derived from uninfected FRE cells transfected with  $D^b$  and  $K^b$ , respectively. EY-10 cells transfected with  $D^b$  yielded clone Df45, while  $K^b$ -transfected EY-10 generated line Q6. FB2 was derived by infecting clone B2 with cloned FMuLV produced by the mouse line  $F_{201}$ NIH. Clone E18 was selected from a culture of SFFV-FRE transfected with  $D^b$  DNA. The results indicate that the transfected murine  $D^b$  and  $K^b$  genes could be strongly expressed in either uninfected or FV-infected FRE cells. In addition,

TABLE I  
*H-2 and Viral Antigen Expression on Virus-infected or -uninfected FRE Clones Transfected with H-2*

Cell line	Components <sup>‡</sup>	Antibody binding* (cpm <sup>125</sup> I bound)			
		28-14-8 anti-D <sup>b</sup>	EH-144 anti-K <sup>b</sup>	55 anti-gp70 <sup>env</sup>	34 anti-p15 <sup>gag</sup>
EL4	H-2 <sup>b</sup> control	2,506	2,398	ND	ND
EY-10n	F + S only	127	151	9,025	1,403
B2	D <sup>b</sup> only	5,341	141	276	176
Df45	F + S/D <sup>b</sup>	2,284	152	7,168	1,530
FB2	F/D <sup>b</sup>	4,421	133	7,508	1,995
E18	S/D <sup>b</sup>	3,693	148	273	511
R19	K <sup>b</sup> only	129	2,829	211	141
Q6	F + S/K <sup>b</sup>	134	1,071	7,828	1,475

\*  $4 \times 10^5$  cells were incubated with diluted monoclonal ascites fluid, washed, and then incubated with <sup>125</sup>I-protein A. Values are mean cpm bound from three replicate wells.

<sup>‡</sup> H-2 and/or viral expression. F, FMuLV; S, SFFV.

<sup>§</sup> Clone EY-10n was selected from a culture of EY-10 that received the selectable marker (pSV2neo) only.

TABLE II  
*Polyclonal Allospecific CTL Recognition of FRE Clones Transfected with H-2*

Target cell line	Components	Percent specific <sup>51</sup> Cr release* from effector populations <sup>‡</sup>		
		Anti-H-2 <sup>b</sup>	Anti-D <sup>b</sup>	Anti-K <sup>b</sup>
EL4	H-2 <sup>b</sup> control	77	82	84
EY-10n	F + S only	6	12	10
B2	D <sup>b</sup> only	42	62	12
Df45	F + S/D <sup>b</sup>	33	51	11
FB2	F/D <sup>b</sup>	47	72	9
E18	S/D <sup>b</sup>	41	63	11
R19	K <sup>b</sup> only	50	9	65
Q6	F + S/K <sup>b</sup>	48	11	58

\* Allospecific CTL were harvested after 6 d incubation in secondary MLC and assayed for cytolytic activity against a panel of <sup>51</sup>Cr-labeled target cells ( $10^4$  cells/well) in a 6-h assay. Values are the mean specific release from three replicate wells.

<sup>‡</sup> E/T ratio = 5:1.

these genes were stably expressed; after one year of continuous culture in selective medium, levels of H-2 antigen similar to those expressed initially were serologically detected on the cell surface (data not shown).

*Allospecific CTL Recognition of H-2-transfected FRE Cells.* Transfected clones were analyzed for recognition by allospecific murine CTL populations in a standard 6-h <sup>51</sup>Cr release assay. The results (Table II) indicate that those cell lines expressing the appropriate H-2 molecule on their surfaces were susceptible to CTL-mediated lysis. Clone EY-10n (EY-10 transfected with pSV2neo only, and therefore not expressing H-2) was not recognized by allospecific CTL; neither were uninfected or SFFV-infected FRE cells that had received pSV2neo

TABLE III  
*Polyclonal Friend Virus-specific CTL Recognition of FRE Clones  
 Transfected with H-2*

Target cell line	Components	Percent specific <sup>51</sup> Cr release from effector populations*	
		Anti-FV complex <sup>‡</sup> (FMuLV + SFFV)	Anti-FMuLV <sup>§</sup>
EY-10n	F + S only	7	7
B2	D <sup>b</sup> only	3	9
Df45	F + S/D <sup>b</sup>	61	46
FB2	F/D <sup>b</sup>	52	64
E18	S/D <sup>b</sup>	31	27
R19	K <sup>b</sup> only	6	43
Q6	F + S/K <sup>b</sup>	9	27

\* Friend virus-specific CTL were harvested after 6 d incubation in secondary MLTC and assayed for cytolytic activity against a panel of <sup>51</sup>Cr-labeled target cells (10<sup>4</sup> cells/well) in a 6-h assay. Values are the mean specific release from three replicate wells.

<sup>‡</sup> E/T ratio = 25:1.

<sup>§</sup> E/T ratio = 75:1.

only (data not shown). No detectable crossreactivity was observed by anti-D<sup>b</sup> CTL against K<sup>b</sup>, or by anti-K<sup>b</sup> effector cells against D<sup>b</sup>. In addition, the degree of lysis (percent specific <sup>51</sup>Cr release) appeared to be independent of the level of cell-surface antigen detected by RIA (compare with Table I). The results thus indicate that no inherent difference in susceptibility to lysis existed among the FRE-derived clones examined.

*FV-specific CTL Recognition of Virus-infected FRE Clones Expressing H-2.* To determine whether the products of the transfected D<sup>b</sup> and K<sup>b</sup> genes could serve as restricting elements for recognition by anti-FV CTL, BALB.B (H-2<sup>b</sup>) mice were immunized with syngeneic, FV-induced tumor cells. These infected stimulator cells express both FMuLV- and SFFV-encoded proteins, as well as endogenous levels of D<sup>b</sup> and K<sup>b</sup>. CTL harvested from secondary restimulation in vitro were used to assay H-2-transfected clones for their susceptibility to lysis in a 6-h <sup>51</sup>Cr release assay. The results (Table III) indicate that the transfected D<sup>b</sup> molecule (in clones Df45, FB2, and E18) could serve as an H-2 restricting element for anti-FV CTL; however, the transfected K<sup>b</sup> molecule (in clone Q6) could not, in agreement with previous studies (2). In addition, cell lines infected with both FMuLV and SFFV (clone Df45), FMuLV only (clone FB2), and SFFV only (clone E18) were susceptible to CTL-mediated lysis. As expected, cells expressing D<sup>b</sup> only (clone B2), K<sup>b</sup> only (clone R19), or both viruses without D<sup>b</sup> (EY-10n) were not recognized by anti-FV CTL. Uninfected FRE, SFFV-FRE, and FMuLV-infected FRE clones that had received pSV2neo only were also not significantly lysed (data not shown).

The results presented in Table III show that both FMuLV- and SFFV-encoded specificities, in association with D<sup>b</sup>, were recognized by anti-FV-directed CTL. The lysis of D<sup>b</sup>-transfected SFFV-FRE cells by these effectors was surprising, as only a small fraction (<10%) of total cellular SFFV-encoded proteins is found on

the surface of infected cells (33). This may account for the relatively reduced level of lysis observed in clone E18 as compared with that seen in FMuLV-infected clone FB2.

We wished to determine whether the polyclonal anti-FV CTL that recognized cells infected with either virus represented separate anti-FMuLV and anti-SFFV effector groups or a crossreacting population. This was accomplished by generating CTL specific for cells from tumors induced with FMuLV alone; i.e., BALB.B mice were immunized with syngeneic, FMuLV-induced tumor cells. FMuLV-induced disease in mice is associated with the formation of a recombinant polytropic virus, Friend mink cell focus-inducing virus (F-MCF) (17, 34, 35), and thus these infected stimulator cells express viral antigens encoded by both FMuLV and F-MCF virus, as well as endogenous levels of  $D^b$  and  $K^b$ . After secondary restimulation in vitro, immune CTL were assayed for their ability to lyse H-2-transfected FRE clones in a 6-h  $^{51}\text{Cr}$  release assay. As shown in Table III,  $D^b$ -transfected cell lines infected with either FMuLV only (clone FB2) or SFFV only (clone E18) were recognized and lysed by the anti-FMuLV CTL population. This finding supports the concept that at least a portion of CTL generated in response to FMuLV-infected cells crossreacted with determinants found on SFFV-transformed cells. Both the *gag*- and *env*-encoded cell-surface proteins of FMuLV and SFFV contain regions of homology (36–38). However, it is uncertain whether the CTL that recognized SFFV-infected clone E18 were initially generated against these homologous FMuLV specificities, or against regions of the F-MCF virus *env* gene product that share extensive homology with those found in SFFV (37, 38).

CTL generated in response to FMuLV-induced tumor cells were also assayed for their ability to recognize and lyse  $K^b$ -transfected cell lines. Unexpectedly, FRE clones expressing  $K^b$  both in the presence (clone Q6) or absence (clone R19) of FV were susceptible to CTL-mediated lysis (Table III). The apparent anti- $K^b$  reactivity of these effector cells suggests that an anti-self CTL response was generated in BALB.B mice exposed to tumor cells initiated by FMuLV. The exact nature of these alloreactive CTL and the role they might play in FMuLV-induced disease has not yet been determined; however, as this phenomenon was not observed with anti-FV-directed CTL, the expression of F-MCF virus in the FMuLV-induced tumor cells used for immunization may play an important role in their generation.

*Separate Expression of the gag and env Genes of FMuLV in FRE Cells.* To identify the individual viral cell-surface molecules recognized by anti-FV CTL, FRE-derived cell lines were constructed that expressed the proteins encoded by either the FMuLV *gag* or *env* genes. This was accomplished by using subcloned genes derived from a molecular clone of viral DNA. Plasmid 2-lalc (Fig. 1) contains a full-length, nonpermuted copy of the FMuLV genome, flanked downstream (3') by a second identical copy of the *gag* gene. This clone was functional when transfected onto uninfected FRE cells; i.e., FMuLV-encoded proteins (Fig. 2) and infectious virions (not shown) were produced. The FMuLV *gag* expression vector, 2-lalc $\Delta$ s, was derived by deletion of the Sac II fragment of plasmid 2-lalc (Fig. 1). This removed the gp70 coding region of *env*, as well as the 5' end of sequences encoding p15(E). The remainder of *env* was restored

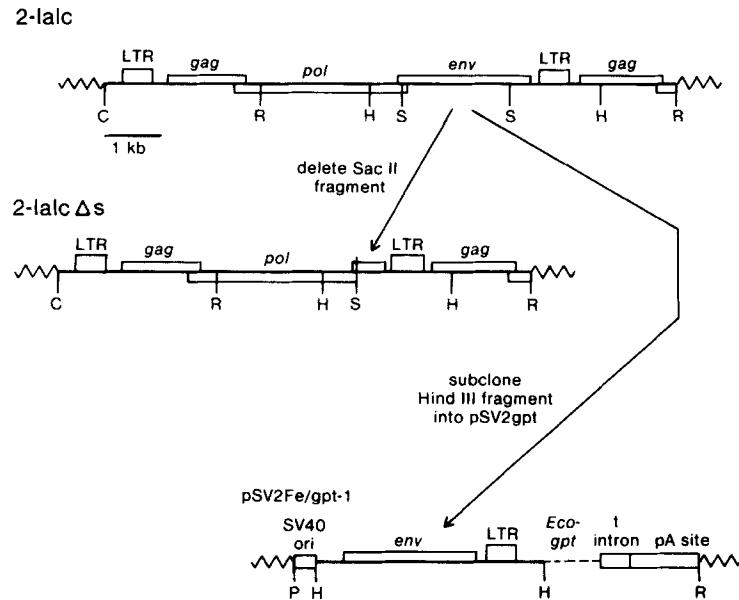


FIGURE 1. Molecular construction of FMuLV subgenomic expression vectors. Plasmid 2-lalc contains a nonpermuted copy of the FMuLV genome. The *gag* expression vector, 2-lalc $\Delta$ s, and the *env* expression vector, pSV2Fe/gpt-1, were derived from 2-lalc as indicated. C, Cla I; R, Eco RI; H, Hind III; P, Pvu II; S, Sac II. Serrated lines represent pBR322 sequences.

in an altered reading frame, leading to premature termination. The *gag* gene, however, remained intact. The expression vector for FMuLV *env*, pSV2Fe/gpt-1, was constructed by subcloning the Hind III fragment of 2-lalc (containing *env* and the 3' viral long terminal repeat [LTR] sequence) into the Hind III site of the eukaryotic expression vector pSV2gpt (39) (Fig. 1). The resulting plasmid thus consisted of the SV40 virus origin and early promoter sequence situated ~750 bp 5' to the start of *env*, which in turn was flanked 3' by the FMuLV LTR. Each of the two subgenomic expression vectors therefore provided the isolated genes with upstream transcriptional promoter sequences, as well as downstream termination and polyadenylation signals.

Uninfected FRE cells were cotransfected with  $D^b$ , pSV2neo, and either 2-lalc $\Delta$ s or pSV2Fe/gpt-1. Cell clones resistant to G418 were analyzed for the expression of  $D^b$  and FMuLV *gag* and *env* products by RIA. Approximately one-half of the clones screened expressed detectable levels of the viral cell-surface protein encoded by the appropriate subgenomic expression vector. Levels of expression varied among the clones, presumably due to the number of vector copies present and their site(s) of integration. Data from representative clones that expressed both  $D^b$  and proteins of individual viral genes at levels similar to those observed in a full viral infection are shown in Table IV. Cell lines EY-10n, B2, and FMuLV-infected FB2 were discussed above, and appear for comparison. Clone N34 was derived from an FRE culture that received pSV2Fe/gpt-1; it expressed  $D^b$  as well as a high level of the *env* product gp70. The presence of p15(E)<sup>env</sup> was also confirmed by the use of a specific monoclonal antiserum directed against that protein (data not shown). No detectable *gag* product was



TABLE IV  
*D<sup>b</sup>* and FMuLV-encoded Antigen Expression on Transfected FRE Clones

Cell line	Components	Antibody binding* (cpm <sup>125</sup> I bound)		
		28-14-8 anti-D <sup>b</sup>	55 anti-gp70 <sup>env</sup>	34 anti-p15 <sup>gag</sup>
EL4	H-2 <sup>b</sup> control	2,352	ND	ND
EY-10n	F + S only	140	8,610	1,316
B2	D <sup>b</sup> only	4,906	252	182
FB2	F/D <sup>b</sup>	4,661	7,408	1,642
N34	F <sub>env</sub> /D <sup>b</sup>	2,626	7,258	161
Ps6	F <sub>gag</sub> /D <sup>b</sup>	2,537	262	1,050

\* As in Table I.

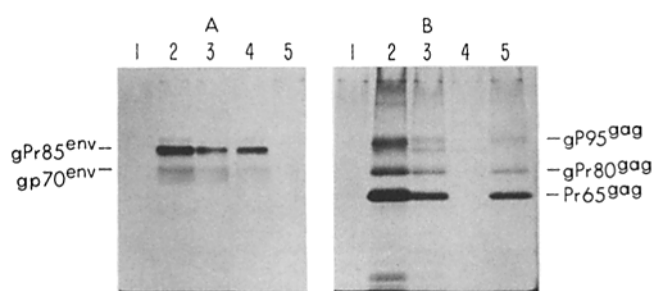


FIGURE 2. Expression of FMuLV-encoded proteins in FRE-derived cell lines. Cells were labeled with [<sup>3</sup>H]leucine for 30 min, and the extracts were immunoprecipitated with goat anti-gp70<sup>env</sup> antiserum (A) or anti-Pr60<sup>gag</sup> antiserum (B). Precipitated proteins were visualized by 7% SDS-PAGE and autoradiography. Cell lines analyzed were uninfected FRE (lanes 1), FMuLV-infected FRE (lanes 2), plasmid 2-lalc (FMuLV)-transfected FRE clone Fc7 (lanes 3), pSV2Fe/gpt-1 (FMuLV *env*)-transfected FRE clone N34 (lanes 4), and 2-lalcΔs (FMuLV *gag*)-transfected FRE clone Ps6 (lanes 5).

observed. Clone Ps6 was selected from a culture transfected with 2-lalcΔs. In addition to D<sup>b</sup>, Ps6 expressed determinants of the *gag*-encoded cell surface proteins gP85 and gP95; no *env* product could be detected. These findings indicate that both of the subgenomic FMuLV expression vectors were functional when transfected into FRE cells, resulting in the expression of separate *gag* and *env* gene-encoded cell-surface proteins.

To verify the identity of the viral cell-surface proteins detected by RIA cell cultures were metabolically labeled with [<sup>3</sup>H]leucine. Viral proteins were immunoprecipitated from the cell extracts with goat antisera reactive with *env*- or *gag*-encoded products and subjected to SDS-PAGE. As shown in Fig. 2, FMuLV-infected FRE cells (lanes 2) synthesized proteins recognized by anti-gp70<sup>env</sup> (Fig. 2A) and anti-Pr60<sup>gag</sup> (B) antisera that were not present in uninfected FRE cells (lanes 1). The *env*-encoded proteins were gp70 and its precursor, gPr85. The products of the *gag* gene were the cell-surface protein gP95 and its precursor, gPr80, as well as the virion core protein precursor Pr65. gP85<sup>gag</sup>, the cell-surface protein derived from gP95, was not detected due to the short (30 min) pulse period used for labeling. A virus-producing cell line, clone Fc7, derived from transfection of FRE cells with plasmid 2-lalc (containing a nonpermuted, full-length FMuLV genome), expressed the same proteins (lanes 3). An anti-*gag*-

TABLE V  
*Polyclonal Friend Virus-specific CTL Recognition of Transfected FRE Clones*

Target cell line	Components	Percent specific <sup>51</sup> Cr release from effector populations*	
		Anti-FV complex <sup>†</sup> (FMuLV + SFFV)	Anti-FMuLV <sup>§</sup>
EY-10n	F + S only	7	5
B2	D <sup>b</sup> only	5	7
FB2	F/D <sup>b</sup>	49	67
N34	Fenv/D <sup>b</sup>	33	42
Ps6	Fgag/D <sup>b</sup>	31	48

\* As in Table III.

<sup>†</sup> E/T ratio = 25:1.

<sup>§</sup> E/T ratio = 75:1.

reacting protein of ~90 kD was also observed in clone Fc7; this may represent the aberrant product of the 3' flanking *gag* gene of 2-lalc due to transcription from the 3' viral LTR (see Fig. 1). This clone was not used in the immunological studies described below.

FMuLV *env*-expressing clone N34 expressed both gp70 and gPr85, but failed to express normal *gag* proteins (Fig. 2, lanes 4). However, a 90 kD protein different from that seen in Fc7 cell extracts reacted weakly with anti-*gag* antiserum. This protein was not detected on the cell surface, nor did it react with antisera specific for the individual *gag* proteins p12, p30, or p10 (data not shown). Thus, it was apparently a hybrid fusion protein that arose by read-through from a brief stretch of p15<sup>gag</sup> coding sequences remaining on the 3' end of pSV2Fe/gpt-1 into *Eco-gpt* sequences. It seems unlikely that this protein could function as a normal *gag* product.

In contrast to clone N34, 2-lalcΔs-transfected clone Ps6 expressed the *gag* proteins Pr65, gPr80, and gP95, while no detectable *env*-encoded products were seen (Fig. 2, lanes 5). It is interesting to note that the 90 kD protein observed in 2-lalcΔs-transfected clone Fc7 was not present; perhaps the Sac II deletion in 2-lalcΔs caused the 3' LTR to become transcriptionally inactive. These results indicate that cell lines N34 and Ps6 expressed normal FMuLV *env* or *gag* proteins only, respectively.

*FV-specific CTL Recognition of FRE Clones Expressing D<sup>b</sup> and FMuLV gag or env.* To determine which viral specificities were required for CTL recognition of FMuLV-infected cells, FRE-derived clones N34 and Ps6 were used as target cells in a 6-h <sup>51</sup>Cr release assay. Virus-specific CTL were generated in BALB.B mice against syngeneic tumors induced either by FV complex or SFFV-free FMuLV as described above. The results (Table V) show that cells expressing either *env*- or *gag*-encoded proteins, in addition to D<sup>b</sup>, were susceptible to lysis by FV-specific CTL. Cell clones expressing FMuLV *gag* or *env* only, in the absence of D<sup>b</sup>, were not lysed by these effectors (data not shown); neither were clones EY-10n (FMuLV + SFFV only) and B2 (D<sup>b</sup> only). As shown above, FMuLV-infected B2 cells (clone FB2) were recognized by anti-FV and anti-

FMuLV CTL. The data thus demonstrate that both the viral *gag* and *env* genes encode specificities that are recognized by anti-FV CTL.

### Discussion

Our analysis of anti-FV CTL specificity involved the use of synthetic target cells, i.e., cell lines that express putative target molecules on a heterologous background. The results show that genes of the murine MHC and discrete retroviral genes can be stably expressed in FRE cells, and that their products have the potential to form cell-surface target structures that can be recognized by virus-specific CTL generated in mice. Cells expressing *H-2D<sup>b</sup>* and either the *env* or *gag* genes of FMuLV were susceptible to lysis by anti-FV CTL; if either the H-2 or the viral components were absent, recognition and lysis did not occur. Experiments with SFFV-infected FRE cells transfected with *D<sup>b</sup>* indicate that the SFFV genome also provides specificities that can form cell-surface target structures recognized by anti-FV CTL.

An important reason for selecting a heterologous cell line, such as FRE fibroblasts, as the parental line for the construction of our synthetic target cells was the expectation that they are more likely than mouse cells to provide an essentially null background for tests of mouse CTL specificity. Individual murine genes can be introduced and stably expressed in a cell-surface environment devoid of other mouse proteins, thus reducing the possibility of nonspecific crossreactions by anti-viral CTL. In addition, any potential crossreactivity of virus-specific CTL with endogenous retroviral proteins expressed by mouse cells is less likely, as FRE cells do not express endogenous retroviral RNA or structural proteins (14). The molecules recognized by murine antiviral CTL can therefore be precisely identified.

Mouse class I MHC genes were stably expressed upon transfection into virus-infected or -uninfected FRE cells, allowing us to separately introduce the K and D class I molecules of the *H-2<sup>b</sup>* haplotype and to analyze the contribution of each to the formation of cell-surface target structures recognized by CTL. In addition, the *H-2K<sup>k</sup>* gene has been successfully expressed in FRE cells (our unpublished results). The introduced H-2 molecules bind appropriate alloantibodies and act as targets for alloantigen-specific mouse CTL. Our results thus extend the findings of other investigators (40), who have shown that mouse allospecific CTL can recognize and lyse human cells that express introduced H-2, indicating that cross-species CTL-target cell interactions can occur. FRE cells have also proved useful for virological studies of FV (15, 16). They are easily and stably infected with virus or transfected with cloned genomic viral DNA, resulting in the normal expression of viral RNA and structural proteins, and they can produce high titers of infectious virus particles. Our data further show that the individual genes of FMuLV, when molecularly cloned into suitable expression vectors, can be correctly and separately expressed in FRE cells.

When expressed in FV-infected FRE clones, the *H-2D<sup>b</sup>* but not the *H-2K<sup>b</sup>* molecule permitted lysis by FV-specific *H-2<sup>b</sup>*-restricted mouse CTL. Previous studies, using target cells obtained from recombinant H-2 mouse strains (2) and analyzing H-2 proteins found in mature viral particles (41, 42) suggested that only the *D<sup>b</sup>* molecule was involved in cell-surface target structure formation.

Our data directly confirm this observation. It is not clear why only  $D^b$  is recognized in association with FV-encoded antigens by CTL generated in mice expressing both  $D^b$  and  $K^b$  molecules. CTL specific for the closely related Moloney and Rauscher MuLV have also been found (43) to be restricted to only the D end of the  $H-2^b$  haplotype. In addition, our demonstration that more than one viral gene product can be recognized in association with  $D^b$  by anti-FV CTL further indicates the dynamic role of the  $D^b$  molecule in target structure formation. While the ability of  $D^b$  and not  $K^b$  to restrict multiple viral proteins may be a coincidence, it is possible that the different structures of the two H-2 molecules may determine a preference for the involvement of the  $D^b$  molecule in anti-FV CTL recognition. Gomard et al. (44) proposed that there exists a hierarchy of H-2 plus viral antigenic associations on the cell surface, with the best available association being immunodominant and thus recognized by CTL precursors. Studies by other investigators (45, 46) showed that the  $D^b$ -restricted anti-Moloney virus CTL response could be transferred to  $K^b$  restriction in mice expressing the  $D^b$  mutation, bm13. However, mice carrying a different mutation in  $D^b$ , bm14, were poor responders, as were recombinant mice expressing only the K end of the  $H-2^b$  haplotype. The CTL response to Moloney virus-encoded antigens in preferential association with  $D^b$  has thus been postulated to reflect an immune response gene function of D region-encoded molecules (44, 45). The D molecule apparently presents viral antigens in association with itself and thereby determines restriction specificity (i.e.,  $D^b$  + viral antigen) in addition to regulating the magnitude of the resulting CTL response (45).

We have also shown that both the FMuLV *env* and *gag* genes, as well as the SFFV genome, provide specificities that are recognized by anti-FV CTL. It thus appears that most if not all of the viral molecules on the cell surface can serve as targets given an appropriate H-2 restricting element, in this case the  $D^b$  molecule. Several reports (10, 12, 13) have implied the existence of CTL populations specific for either *env* or *gag* gene products of Moloney MuLV. Gross MuLV-specific CTL were shown (47) to lyse mouse L cells transformed with the *gag* gene of the endogenous ecotropic MuLV, Akv. With respect to FV, the FMuLV-encoded protein gp70<sup>env</sup> has been suggested (11) as a target molecule for anti-FV CTL. In addition, CTL directed against SFFV-specific antigenic determinants can be generated in mice immunized with syngeneic SFFV-infected nonproducer cells (48). However, the relatively high level of lysis of  $D^b$ -transfected SFFV-FRE cells we observed was surprising, as the FV-infected tumor cells used to generate CTL expressed substantially more FMuLV- than SFFV-encoded cell-surface proteins (33 and Table I). This observation may be due to the existence of crossreactive CTL that recognize determinants of both FMuLV and SFFV. The finding that CTL generated against FMuLV-induced tumor cells can lyse target cells expressing only SFFV antigens shows that crossreaction can occur. However, a question left unresolved by our studies concerns the distinction between antigenic determinants that are specific to individual viruses involved in FV-associated diseases and those shared by the viruses. Analysis of this question is complicated by the involvement of a third virus, F-MCF virus, in certain of these diseases, in addition to the more classical FMuLV and SFFV. F-MCF appears to play a routine and probably necessary role in the generation of erythroleukemias

by SFFV-free FMuLV (17, 34, 35), and it may also play a role in the late stages of the SFFV-associated disease as well. Some antigenic determinants will probably prove to be shared by all three viruses. Also likely are crossreactions between those *env* gene segments of SFFV and F-MCF virus that are unrelated to the FMuLV *env* gene. F-MCF virus is generated *de novo* in the development of each FMuLV-induced tumor, and to date there has been no analysis of the extent to which separate F-MCF virus isolates can differ from each other.

The question of crossreactive vs. individual virus-specific antigenic determinants arises in two contexts from our studies. First, the ability of anti-FV complex CTL to lyse FRE cells expressing either FMuLV alone or SFFV alone could be due to recognition of both crossreactive and individual virus-specific determinants. The ability of anti-FMuLV CTL to lyse cells expressing only SFFV antigens could similarly arise either from crossreactions between FMuLV- and SFFV-encoded determinants, or from those between SFFV and F-MCF determinants. The second context concerns the unexpected finding that anti-FMuLV CTL but not anti-FV CTL appear capable of lysing cells on the sole basis of the presence of the  $K^b$  gene product. While other possible explanations of this phenomenon have not been ruled out, we are particularly interested in the hypothesis that the  $D^b$  molecule can be modified by interaction with an F-MCF viral molecule on the FMuLV-induced tumor cell surface and thus acquire one or more  $K^b$ -like determinants. This might result in the generation of crossreactive CTL that recognize the virus-altered  $D^b$  molecule as well as the unmodified  $K^b$  molecule. Studies in progress with cloned CTL populations should help resolve these questions.

### Summary

We have constructed a series of "synthetic" target cell lines for an analysis of the specificity of anti-Friend virus (FV) CTL. Our results show that murine H-2 genes and individual retroviral genes can be stably expressed in Fisher rat embryo (FRE) cells, and that their products have the potential to form target structures recognized by mouse CTL. Cells expressing  $H-2D^b$  and either the *env* or *gag* genes of one component of FV, helper Friend murine leukemia virus (FMuLV), were lysed by anti-FV CTL and by CTL generated against FMuLV alone. Experiments with  $D^b$ -transfected FRE clones infected only with the replication-defective spleen focus-forming virus (SFFV) component of FV indicate that the SFFV genome also provides specificities recognized by both anti-FV and anti-FMuLV CTL, thus demonstrating the existence of a crossreactive CTL population. An unexpected finding was that anti-FMuLV CTL, but not anti-FV CTL were also able to lyse FRE clones that expressed  $H-2K^b$  in either the presence or absence of FV. The use of heterologous cell lines for the construction of synthetic target cells thus offers a useful approach for the analysis of T cell specificity.

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