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# Molecular Cloning of Infectious Ecotropic Murine Leukemia Virus AK7 from an *emv-14*-Positive AKXL-5 Mouse and the Resistance of AK7 to Recognition by Cytotoxic T Lymphocytes

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The AKXL-5 recombinant inbred mouse strain is positive for the endogenous ecotropic murine leukemia virus *emv-14*, the only *emv* present in its germ line. *emv-14* is of particular interest because spleen cells expressing *emv-14* virus escape recognition by anti-AKR/Gross virus-specific cytotoxic T lymphocytes. We report here the isolation and characterization of a replication-competent *emv* clone, pAK7, derived from an AKXL-5 mouse. This clone is novel in that it encodes a variant ecotropic murine leukemia virus that, when expressed in SC.K<sup>b</sup> target cells, fails to be recognized efficiently by anti-AKR/Gross virus cytotoxic T lymphocytes. The pAK7 clone can therefore be used to further probe mechanisms of escape from cell-mediated immunity.

The AKR mouse strain has a high incidence of leukemia that, although proximally caused by recombinant mink cell focus-forming (MCF) murine leukemia virus (MuLV), is dependent on the expression of endogenous ecotropic MuLV (EMV) (22, 26). It has been shown that an *H-2K<sup>b</sup>*-restricted cytotoxic T-lymphocyte (CTL) response can be raised against tumor cells expressing EMV in C57BL/6 mice (8, 11), which have a low incidence of leukemia. We have designated these effector cells anti-AKR/Gross virus CTL.

The AKXL recombinant inbred strains (34), which were generated from the AKR (*H-2<sup>k</sup>*, positive for *emv-11*, *emv-13*, and *emv-14*) and the C57L (*H-2<sup>b</sup>*, *emv* negative) progenitor strains, were used to characterize the specificity of anti-AKR/Gross virus CTL (10). Only spleen cells from AKXL strains that were *H-2<sup>b</sup>* and *emv-11* positive could stimulate an anti-AKR/Gross virus CTL response from tumor-primed C57BL/6 responder cells. AKXL spleen cells that were positive only for *emv-14* or for *emv-14* and the replication-defective *emv-13* could not stimulate a response. Correspondingly, SC.K<sup>b</sup> (*H-2K<sup>b</sup>*) fibroblasts infected with *emv-14* virus isolated from an AKXL-5 mouse (*emv-14* positive only) and used as targets in chromium-51 release assays were not recognized by anti-AKR/Gross virus CTL (38). These findings were curious, since *emv-14* is replication competent and expressed in a seemingly normal fashion. The fact that *H-2*-compatible fibroblasts expressing virus from AKXL-5 mice are not lysed by anti-AKR/Gross virus CTL indicates that a mutation within the *emv-14* provirus results in a loss of CTL recognition. In addition, the defect must lie at the level of target cell recognition rather than at some earlier step, such as T helper cell function.

In order to further characterize this failure of CTL to recognize AKXL-5 EMV, we cloned a replication-competent *emv*, pAK7, from AKXL-5 genomic DNA. Clone pAK7 is characterized and compared with pAKR623 (23), a provirus that is derived from EMV-11. SC.K<sup>b</sup>/623 fibroblast cells

expressing AKR623 virus are lysed efficiently by anti-AKR/Gross virus CTL (38). We show here that SC.K<sup>b</sup>/7 cells expressing virus from pAK7 are not lysed efficiently by anti-AKR/Gross virus CTL. The pAK7 clone therefore affords us the opportunity to study a mechanism by which a retrovirus may escape destruction by antiviral CTL.

**Isolation of clone pAK7 from AKXL-5 genomic DNA.** DNA (obtained from Ben Taylor, The Jackson Laboratory) from the spleen cells of an AKXL-5 mouse (*emv-14* positive, *emv-11* negative) was *EcoRI* restricted, and a fraction of 11 to 15 kb was used to construct a phage lambda library by using the replacement vector EMBL-4. After identifying positive clones with the *emv*-specific probe, which detects only ecotropic DNA sequences and not xenotropic, amphotropic, or MCF MuLV (2, 20), the clone λAK7 was grown by standard procedures (33, 39) and subcloned into the *EcoRI* site of pBR322 for use in further studies.

**Analysis of pAK7 by restriction endonuclease mapping.** Figure 1 shows the restriction map of pAK7. Approximately 0.5 kb of 5'-flanking murine sequences and 3.5 kb of 3'-flanking murine sequences surround the pAK7 provirus on the *EcoRI* fragment cloned. All of the restriction sites present in pAKR623 (sequence data are in references 14 and 37) that were tested for were found in equivalent locations in pAK7. The pAK7 provirus has the hallmarks of an *emv*: (i) it hybridizes with the *emv*-specific DNA probe; (ii) the mapping data confirmed the expected *SmaI* and *KpnI* sites within the provirus; (iii) *PstI* sites were found only within the long terminal repeats (LTRs), as expected (some xenotropic viruses have a *PstI* site in the *pol* region which is not present here and is never seen in *emv* proviruses [1], and there is a *PstI* site in the *env* region of xenotropic and endogenous polytropic viruses which is not present in clone pAK7 and is never seen in *emv* viruses [1, 15, 16]); (iv) noncancerous endogenous proviruses all contain an *EcoRI* site in the *env* region (1, 15, 35, 36), but there is none within the clone pAK7 provirus or within *emv* proviruses; and finally, (v) the expected *emv*-specific *XbaI* site within the p15E coding portion of *env* is seen in clone pAK7, this site being absent

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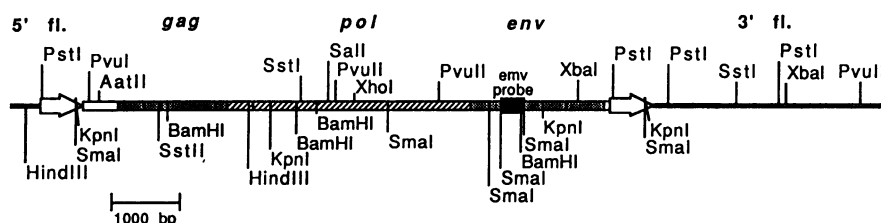


FIG. 1. Restriction map of clone pAK7. The fragment cloned and represented here is bounded by *EcoRI* sites. fl., flanking; arrows, LTRs.

from xenotropic (16) and endogenous polytropic MuLV (3). Thus, pAK7 represents an ecotropic provirus clone, not an endogenous xenotropic or polytropic MuLV.

**Identification of an RFLP in pAK7 relative to pAKR623.** After close inspection of restriction digests of pAK7 and comparison with those of the *env*-11-like clone pAKR623 (kindly provided by S. K. Chattopadhyay [23] through B. A. Taylor), a restriction fragment length polymorphism (RFLP) was found in the LTR region (Fig. 2). Both the *SmaI* and the *KpnI* fragments corresponding to the 3' *env*/LTR region of pAK7 are shortened by approximately 100 bases compared with the fragments from pAKR623. By doubly digesting each of the two clones with *KpnI* and *PstI*, the location of the RFLP was further defined as being within the U3R region of the LTR. It is noteworthy that the direct repeat in the enhancer region of the U3 portion of the LTR is approximately 100 bp long and is duplicated in pAKR623. Similar

polymorphisms in the LTR have been shown by others (29, 30).

**Expression of AK7 virus in fibroblast cells.** In order to characterize the virus derived from the pAK7 clone, SC.K<sup>b</sup> cells (38) were used to express AK7 virus. SC.K<sup>b</sup> cells are *env* negative and express the appropriate major histocompatibility complex (MHC) restricting element for use as a target cell line for *H-2K<sup>b</sup>*-restricted anti-AKR/Gross virus CTL. The  $\Psi$ CRIP cell line (kindly provided by Richard Mulligan [6]) was used to pseudotype the virus to facilitate infection into SC.K<sup>b</sup> cells.

SC.K<sup>b</sup> cells were infected with AK7 virus or with AKR623 virus for comparison, resulting in the SC.K<sup>b</sup>/7 and SC.K<sup>b</sup>/623 cell lines, respectively. Cell surface viral antigen expression was analyzed by indirect immunofluorescence and fluorescence-activated cell sorting (FACS) analyses. The monoclonal antibody 24-8 (mouse immunoglobulin G2a [IgG2a]) binds to the *env* gp70 envelope antigen (28). The monoclonal antibody 548 (mouse IgG2b) binds p12<sup>gag</sup> antigens (4). Figure 3 shows the results after a period of weeks, when virus expression had reached steady-state levels. It can be seen that the levels of expression of gp70 detected by the anti-gp70 monoclonal antibody 24-8 (fluorescence intensity measured with a linear amplifier) are similar for both SC.K<sup>b</sup>/7 and SC.K<sup>b</sup>/623 cells, while there is no expression of EMV-specific gp70 on uninfected SC.K<sup>b</sup> cells. The levels of anti-p15E<sup>c</sup> (determined with antibody 19-F8 [25]; data not shown) and anti-p12<sup>gag</sup> antibody binding are also similar for SC.K<sup>b</sup>/7 and SC.K<sup>b</sup>/623 cells. Other monoclonal antibodies against various *gag*- and *env*-encoded epitopes gave similar results as well (data not shown). In some FACS analyses, the levels of viral antigen expression were slightly greater on SC.K<sup>b</sup>/7 cells than on SC.K<sup>b</sup>/623 cells. The two cell lines described here were chosen for further CTL analyses (below) because of their consistent expression of nearly equal levels of cell surface viral antigens.

SC.K<sup>b</sup>/7 cell supernatant fractions were positive when assayed for reverse transcriptase activity (31), with levels similar to those observed for SC.K<sup>b</sup>/623 supernatant fractions (data not shown). In summary, steady-state levels of expression of AK7 *gag*-, *pol*-, and *env*-encoded antigens are essentially equivalent to those of AKR623 antigen expression. RNA blot analysis with the *env*-specific probe against RNA isolated from SC.K<sup>b</sup>/7 cells confirmed that pAK7 encodes an ecotropic MuLV (data not shown).

**SC.K<sup>b</sup>/7 target cells are lysed poorly by anti-AKR/Gross virus CTL.** The SC.K<sup>b</sup>/7 cells were then assayed at the target level against anti-AKR/Gross virus CTL. These *H-2K<sup>b</sup>*-restricted C57BL/6 CTL were raised by *in vivo* priming and secondary *in vitro* stimulation with MHC-compatible AKR/Gross virus-induced tumor cells as described previously (11, 27). Each chromium release experiment was performed in

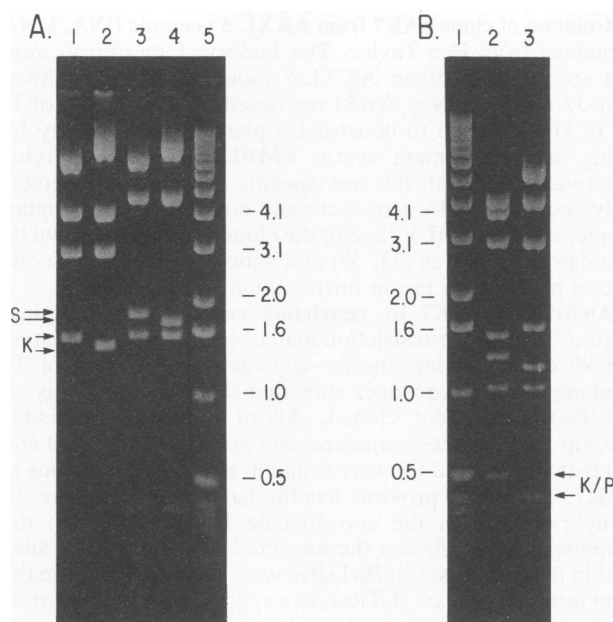


FIG. 2. Ethidium bromide-stained gel, showing the RFLP of clone pAK7 relative to pAKR623. (A) Lanes 1 and 3, pAKR623; lanes 2 and 4, pAK7; lanes 1 and 2, digestions with *KpnI*; lanes 3 and 4, digestions with *SmaI*. (B) Lane 2, pAKR623 doubly digested with *KpnI* and *PstI*; lane 3, pAK7 doubly digested with *KpnI* and *PstI*. Lanes A5 and B1 contain size standards (in kilobases). The arrows point to the *SmaI* (S) and *KpnI* (K) RFLPs in panel A and to the *KpnI*-*PstI* (K/P) RFLP in panel B. Other differences in banding between pAK7 and pAKR623 are due to differences in the flanking sequences (see Fig. 1).

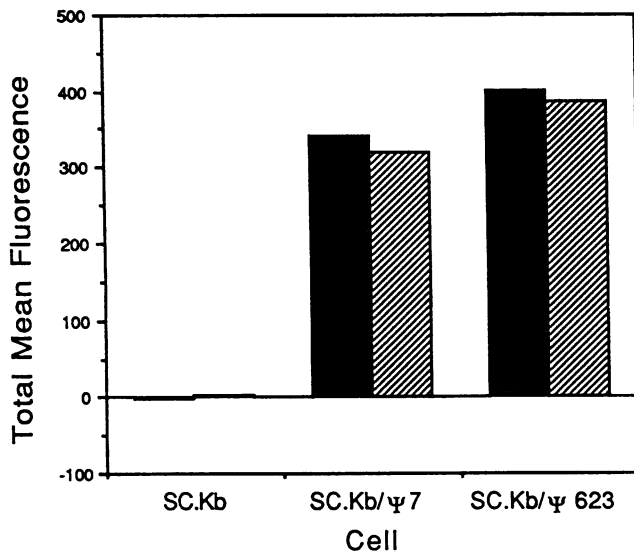


FIG. 3. FACS analysis of the expression of EMV on SC.K<sup>b</sup> cells infected with AK7 or AKR623 virus. The pAK7 or pAKR623 plasmid was used along with plasmid pSVhis<sup>+</sup> (obtained from R. Mulligan), carrying the dominant selectable His<sup>+</sup> marker to transfect ΨCRIP cells by the calcium phosphate DNA coprecipitation method (7) to generate the ΨCRIP7 and ΨCRIP623 cell lines, respectively. Cell-free supernatant fractions were used to generate the SC.K<sup>b</sup>/7 and SC.K<sup>b</sup>/623 lines, respectively, from Polybrene-treated (20 μg/ml) SC.K<sup>b</sup> cells. The expression of EMV on these cells was analyzed by cytofluorography and indirect immunofluorescence with monoclonal antibodies against gp70 (24-8, shaded bars) and p12 (548, hatched bars) (see text). The staining procedure used has been described before (38). Background fluorescence values were determined with the following irrelevant monoclonal antibodies used as isotype controls: OKT8 (CRL 8014, anti-CD8 mouse IgG2a) for 24-8 and OKM1 (CRL 8026, mouse IgG2b, against the integrin C3bi receptor on monocytes) for 548. The secondary antibody used was fluorescein isothiocyanate-labeled goat anti-mouse polyvalent F(ab')<sub>2</sub> (Cappel). Nonspecific fluorescence was subtracted from the fluorescence values, measured with a linear amplifier. Flow cytometry analysis was performed on an Ortho 50H cytofluorograph.

duplicate and with two independent sets of stimulated effectors. This experiment was performed three times with similar results. SC.K<sup>b</sup>/7 targets are lysed by these CTL at lower levels than SC.K<sup>b</sup>/623 targets (Fig. 4). Thus, clone pAK7 encodes an EMV that fails to be recognized efficiently by anti-AKR/Gross virus CTL, similar to the low response by these CTL against spleen cell targets expressing *emv-14* (10). The poor recognition of SC.K<sup>b</sup>/7 targets occurs despite similar steady-state levels of *gag*-, *pol*-, and *env*-encoded viral antigens for SC.K<sup>b</sup>/7 and SC.K<sup>b</sup>/623 (see above).

**Clone pAK7 encodes replication-competent virions.** Because the previous assays were insufficient to determine whether pAK7 encoded infectious, replication-competent virus, the standard XC plaque assay (21, 32) was performed. No virus could be detected for SC.K<sup>b</sup>/7 cells by this method, even when repeated four times over a period of months, whereas titers were typically in the 10<sup>5</sup> to 10<sup>6</sup> PFU/ml range for SC.K<sup>b</sup>/623 cells. The lack of XC positivity was curious because earlier experiments with extracts from tail tissue from AKXL-5 mice, which carry only *emv-14*, showed that this strain was viremic by the XC plaque assay (34, 38). Some possible explanations for this discrepancy are: (i) pAK7 encodes a replication-defective virus; (ii) pAK7 en-

codes a replication-competent virus that is not detectable on the basis of overt plaques by the XC plaque assay; and (iii) XC-positive virus detected in AKXL-5 mice is an XC-positive derivative of EMV-14, and virus expressed directly from the *emv-14* locus is XC negative.

To distinguish among these possibilities, a virus transfer experiment was done. Cell-free virus (unaided by helper virus) from SC.K<sup>b</sup>/7 or SC.K<sup>b</sup>/623 cells was used to infect fresh virus-negative SC.K<sup>b</sup> cells. When the resultant SC.K<sup>b</sup>/7<sub>P2</sub> (secondary passage) cells were assayed for virus expression by FACS analysis over time, the level of expression of gp70 and p12 antigens was lower at first than in SC.K<sup>b</sup>/623<sub>P2</sub> cells but approached high steady-state levels by 4 to 6 weeks after infection (Fig. 5). The initial rate of virus expression for the secondary-passaged AK7 was also similar to that for the primary-passaged AK7 (data not shown). In contrast, the expression of virus on SC.K<sup>b</sup>/623<sub>P2</sub> cells was maximal by 1 week postinfection, the earliest time point measured. These experiments indicate that pAK7 does encode infectious virus, although the initial rate of appearance of viral antigens after infection is more rapid for pAKR623-encoded virus (selected for its ability to spread rapidly) than for pAK7-encoded virus.

When SC.K<sup>b</sup>/7 cells (primary passage) were cocultured in equal numbers with XC cells and the plates were stained 24 and 48 h later, giant multinucleated cells were observed in numbers similar to those seen for SC.K<sup>b</sup>/7<sub>P2</sub> and SC.K<sup>b</sup>/623 in parallel assays. SC.K<sup>b</sup> cells failed to induce giant multinucleated cells, as expected. This experiment was performed twice with the same results. Thus, although SC.K<sup>b</sup>/7 cells appear to be XC plaque assay "negative," at least as defined by the presence of obvious plaques in the standard assay, they are XC positive in a cocultivation assay, indicating the presence of functional virus.

Interestingly, when SC.K<sup>b</sup>/7<sub>P2</sub> cells were assayed by the standard XC plaque assay, they scored positive in two of two experiments, each in triplicate, indicating that the AK7 virus had acquired overt XC positivity after passage. Nonetheless, these SC.K<sup>b</sup>/7<sub>P2</sub> cells still failed to be lysed efficiently by bulk antiviral CTL despite their XC positivity. At an effector-to-target cell ratio of 100:1, 95% of SC.K<sup>b</sup>/623 targets were lysed, whereas SC.K<sup>b</sup>/7<sub>P2</sub> targets and uninfected SC.K<sup>b</sup> targets were lysed at similar low levels (22 and 16%, respectively). Thus, the mutation responsible for apparent XC negativity is separate from the mutation(s) responsible for low levels of lysis by CTL. The secondary virus passage data also confirm the original finding (38) that the integration of virus into new sites within the murine genome, as shown here for SC.K<sup>b</sup>/7<sub>P2</sub>, fails to alter the CTL phenotype, indicating that proviral flanking sequences are not responsible for the low levels of lysis by CTL.

It is unlikely that the slower appearance of viral antigens following de novo infection is responsible for the poor recognition by CTL, since CTL assays were done when viral antigens were at steady-state levels and those levels were roughly equivalent between SC.K<sup>b</sup>/7 and SC.K<sup>b</sup>/623 cells. Furthermore, the two target cell lines should not differ in antigen processing or presentation, since the same SC.K<sup>b</sup> line was used for each infection. Although we think it unlikely that the rate of virus spread for AK7 contributes to poor recognition by CTL, future studies with a chimera of pAK7 containing the pAKR623 LTRs would be useful for resolving this possibility.

**Clone pAK7 represents an *emv-14* reintegration locus.** Because clone pAK7 encodes replication-competent EMV and because the source of DNA used for constructing the library

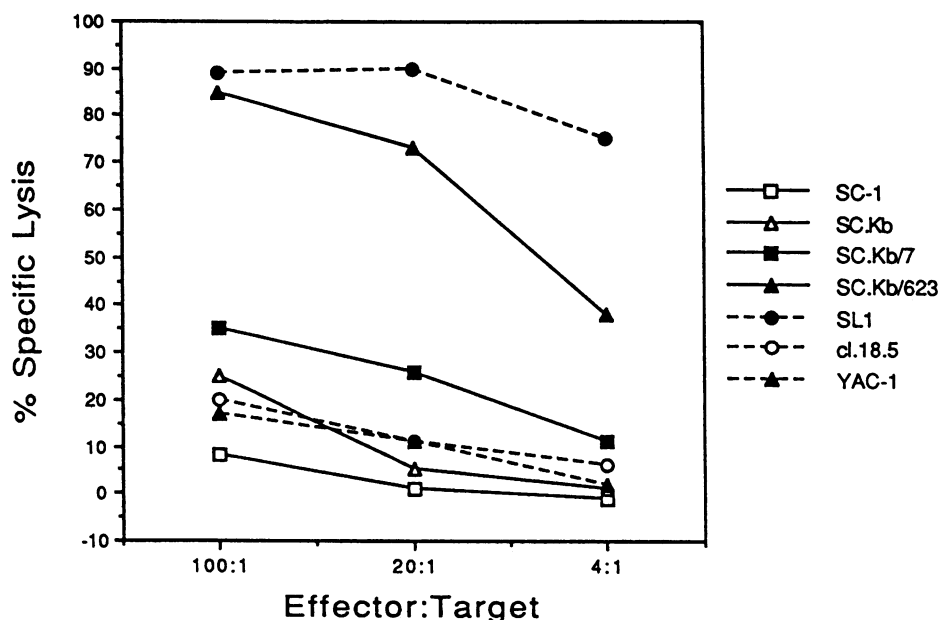


FIG. 4. Failure of anti-AKR/Gross virus CTL to lyse SC.K<sup>b</sup>/7 target cells. CTL were raised as described in the text and previously (38). Data for CTL stimulated with irradiated E $\delta$ G2 (derived from a C57BL/6 tumor induced with AKR/Gross virus passage A [11]) effectors are shown here. Control fibroblast cells (unbroken lines) were SC-1 (EMV negative, derived from a wild mouse embryo [12]), SC.K<sup>b</sup> (*H-2<sup>b</sup>* [38]), and SC.K<sup>b</sup>/623 (SC.K<sup>b</sup> cells infected with AKR623 EMV). Control tumor cells (dashed lines) were SL1 (AKR.H-2<sup>b</sup>-derived spontaneous tumor cell); clone 18-5, a variant line derived from SL1 that is only poorly recognized by anti-AKR/Gross virus CTL (9); and YAC-1, an *H-2<sup>a</sup>* tumor cell line that is used here as a highly natural killer cell-sensitive target cell to control for natural killer cell killing.

from which pAK7 was isolated carried only *emv-14*, clone pAK7 must ultimately be derived from the *emv-14* locus. Southern blot analysis with both *emv*-specific and pAK7 flanking cellular DNA probes indicated that the provirus

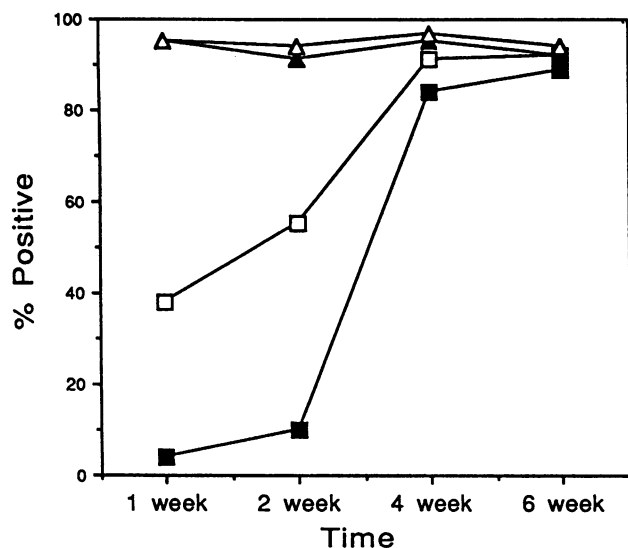


FIG. 5. Time course of cell surface virus expression after secondary passage of virus. FACS analysis (as in Fig. 3) showing the rates of appearance of virus expression from a secondary-infection experiment in which SC.K<sup>b</sup> cells were infected with the supernatant fractions from SC.K<sup>b</sup>/7 or SC.K<sup>b</sup>/623 (Fig. 3), resulting in SC.K<sup>b</sup>/7<sub>P2</sub> and SC.K<sup>b</sup>/623<sub>P2</sub>, respectively. Solid symbols, 24-8 (anti-gp70); open symbols, 548 (anti-p12); squares, SC.K<sup>b</sup>/7<sub>P2</sub>; triangles, SC.K<sup>b</sup>/623<sub>P2</sub>.

cloned represents a reintegration site rather than the *emv-14* locus per se (data not shown). The genomic DNA used to construct the library from which  $\lambda$ AK7 was cloned was isolated from a 1.5-year-old mouse (these mice are now extinct) and therefore probably contains new proviral integrations as a result of reinfection by EMV expressed from the *emv-14* locus. It is therefore likely that pAK7 represents a genomic clone of one such reintegrated, *emv-14*-derived provirus.

Regarding the slower rate of appearance of virus expression after infection with AK7 than after infection with AKR623, it should be noted that AKR623 was isolated after multiple passages that would select viruses that replicate most efficiently (23). One reason for the differences in the rates of appearance of virus expression demonstrated here between AK7 and AKR623 may be in the U3 enhancer region of the LTR. AKR623 has a direct repeat (a duplication of about 100 bp), and it is well known that, in general, multiple enhancers are more effective at increasing transcription rates than single copies. It is interesting, then, that pAK7 has a deletion (or rather a lack of duplication) of approximately 100 bp in this region relative to pAKR623, and this RFLP may reflect a single 100-bp enhancer-like direct repeat rather than the duplication seen in pAKR623. Examples of *emv* proviruses with a single direct repeat include *emv-1* (18) and *emv-3* (19). There are other examples of LTR polymorphisms between *emv*'s as well (29, 30).

It is also possible that the single direct repeat in the U3 region of pAK7 is partly responsible for the inability of pAK7-encoded virus to score positive in the XC plaque assay, if the kinetics of the AK7 replication cycle are too slow to allow sufficient virus spread within the time frame of the assay. Yet the overtly XC-positive SC.K<sup>b</sup>/7<sub>P2</sub> line exhibited a slow initial appearance of viral antigens, similar to that

with SC.K<sup>b</sup>/7. An alternative explanation is that AK7 has an altered determinant that is involved in syncytium formation, as may be the case for XC-negative infectious MuLV found by others. For example, neither MCF-infected mink cell cultures nor MCF-infected mouse cell cultures develop syncytia when cocultivated with XC cells (13). There are also N-tropic MuLV variants that are infectious but XC plaque assay negative (5, 17, 24). Whatever mutation(s) is responsible for the XC negativity (as defined by the absence of obvious plaques), the XC phenotype becomes positive upon secondary passage of the AK7 virus and thus does not correlate with the dysfunctional CTL phenotype.

In summary, we have cloned and characterized an infectious EMV variant, AK7, and cells expressing this immunologic variant fail to be recognized efficiently by anti-AKR/Gross virus CTL. SC.K<sup>b</sup>/7 cells can now be used to further define the viral epitope(s) that is missing or altered in AK7 relative to AKR623. In addition, AK7 virus-positive cells can be used to determine whether AK7-specific CTL can be induced, and the mechanism by which this virus evades CTL recognition can be studied further. Such evasion of cellular immunity, a primary defense against retroviral infection, is one way in which virus-induced tumors may escape immune surveillance and become established.

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