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Molecular Properties of a gag^- pol^- env^+ Murine Leukemia Virus from Cultured AKR Lymphoma Cells

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We have described the isolation of a replication-defective murine leukemia virus from a culture of AKR lymphoma cells [Rein et al., Nature (London) **282**:753–754, 1979]. To facilitate the characterization of this murine leukemia virus, we transmitted it to mink cells and analyzed its genome by restriction mapping of the mink cellular DNA. This genome resembled the *Akv* genome quite closely, but it had an additional *Kpn*I cleavage site at 1.3 kilobase pairs from the 5′ end of the provirus and a small (~50-base-pair) deletion between 1.8 and 3.0 kilobase pairs from the 5′ end. When we tested these mink cells by immune precipitation or by competition radioimmunoassay, we found that they synthesized $gPr82^{env}$, but contained no detectable *gag* or *pol* proteins. It seems likely that the *Kpn*I cleavage site at 1.3 kilobase pairs reflects an abnormal sequence at or near the beginning of the *gag* gene, which prevents *gag* or *pol* translation by introducing a frameshift or termination codon into this region.

Genetic variants of viruses are of fundamental importance in analyzing the roles of individual gene products in viral life cycles. However, relatively few variants of the mammalian retroviruses have been characterized in detail. We have described the isolation of a non-conditionally defective murine leukemia virus (MuLV) from a culture of AKR lymphoma cells (21). The lesion in this MuLV appeared to be quite severe; although the virus was detected by its ability to induce XC plaque formation in the presence of an appropriate helper virus (the complementation plaque assay) (22), cells containing the defective MuLV contained no virus particles or clear virus-specific structures when they were examined by electron microscopy (21).

This defective MuLV is also unique in that it was obtained from cultured AKR lymphoma cells. AKR mice possess an endogenous ecotropic MuLV, designated *Akv*, which is expressed at high levels throughout the life of the mice (27) and appears to play some causative role in the development of spontaneous thymic lymphomas in these mice (5, 7, 10, 14, 15). Since *Akv* is produced at high levels by normal AKR tissues and cell lines (27), it was surprising that it was not produced by cultures of AKR lymphoma cells (16, 17). These findings raised the question of the origin of the defective MuLV. One possibility is that it is a previously undescribed endogenous virus which is inherited independently from *Akv* and is expressed specifically in thymic or leukemic cells; alternatively, it might be derived from *Akv* in the somatic tissues of the mouse by mutation or recombination. Furthermore, the defectiveness of this virus might represent a substitution of nonviral sequences, as in the mammalian sarcoma viruses.

In this paper we present a partial molecular characterization of the defective MuLV. This virus apparently synthesizes an *env* protein but no *gag* or *pol* gene product. The restriction map of the defective provirus is quite similar to that of *Akv*, and our results are fully consistent with the hypothesis that the defective virus arose from *Akv* while replicating in the somatic tissues of the mouse. One difference from *Akv*, which occurs in the extreme 5′ portion of the *gag* gene, may be responsible for the lack of detectable *gag* or *pol* protein synthesis.

MATERIALS AND METHODS

Cells and viruses. The general tissue culture procedures used in this study have been described previously (24). The S^+L^- focus assay, which detects all nondefective MuLV's that replicate on mouse cells, and the complementation plaque assay, which detects replication-defective as well as nondefective ecotropic MuLV's by using the XC test in the presence of an XC-negative helper virus, were performed as reported previously (2, 22), except that the helper virus used in the complementation plaque assay was Moloney clone 83 (21).

The AKR2B cell line (26), a line of AKR mouse embryo fibroblasts which contain the *Akv* genome but do not normally produce virus, was obtained from Sisir K. Chattopadhyay, National Cancer Institute (NCI). Mink cell clone CCL64 was obtained from Paul Peebles (NCI) and was grown in Dulbecco modified Eagle medium containing 10% fetal calf serum.

Feline leukemia virus (subgroup C) was a gift from Charles D. Sherr (NCI); baboon endogenous virus was obtained from chronically infected Cf2Th cells, which were kindly supplied by Ellen Cusick and George Todaro (NCI).

A clone of mink cells that were productively infected with WN1802N MuLV (BALB/c-S2N MuLV) (6) was originally isolated by P. O. Weislogel and R. H. Bassin (unpublished data). The mink cells were infected with a phenotypically mixed stock of WN1802N MuLV which also contained xenotropic MuLV, and then cloned 1 day after infection. One clone isolated in this experiment produced ~10^5 XC plaque-forming units of N-tropic MuLV per ml, with a purely ecotropic host range. This clone was used as a control in all of the experiments described below. Since identical maps for WN1802N and *Akv* were derived in a recent study in which 12 restriction endonucleases were used (20), for convenience we refer to this clone as mink-(*Akv*) below. Although the DNA of this clone contained all of the restriction fragments expected in the *Akv* genome, we also detected additional fragments. It appears that at least one other MuLV-related genome, possibly a defective derivative of xenotropic MuLV, was also present in this cell line.

Molecular hybridization. Cellular DNA was prepared by phenol-chloroform extraction, spooled out of ethanol, treated with RNase, and reextracted with phenol and chloroform. Restriction endonuclease digestions, agarose gel electrophoresis, transfer to nitrocellulose membranes, hybridization to [^{32}P]DNA probes, and autoradiography were performed as described previously (20, 29). ^{32}P-labeled AKV probes were synthesized by nick-translation, using *Escherichia coli* polymerase and DNase (13), from plasmids containing the 8.2-kilobase-pair (kbp) *Pst*I fragment of *Akv* clone 623 (12) (i.e., a fragment extending from 0.1 to 8.3 kbp on the 8.8-kbp *Akv* genome) or the 1.9-kbp *Bam*HI fragment of clone 623 (i.e., the fragment between 1.85 and 3.7 kbp on the *Akv* map) inserted into pBR322 (20). The latter probe was a generous gift from Malcolm A. Martin, National Institute of Allergy and Infectious Diseases. Under the conditions which we used, fragments of high-molecular-weight DNA which were smaller than 0.7 kbp were not visualized routinely.

Radioimmunoprecipitation. All radioisotopes were purchased from New England Nuclear Corp., Boston, Mass. Cells were labeled in minimal essential medium lacking labeled amino acid but supplemented with 1% dialyzed fetal calf serum. After labeling, the cells were rinsed with cold phosphate-buffered saline and scraped into lysis buffer (0.02 M sodium phosphate, pH 7.5, 0.1 M NaCl, 0.001 M EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate). Then they were passed through a 19-gauge needle, and the extract was clarified by centrifugation at 6,000 rpm for 20 min in a Sorvall HS-4 rotor. The resulting supernatant was incubated for 3 h with normal goat serum and Formalin-fixed *Staphylococcus aureus*; this mixture was centrifuged for 3 h at 40,000 rpm in a Beckman 50Ti rotor.

A total of 2 × 10^6 acid-precipitable counts was precipitated with goat antisera to Rauscher MuLV proteins (provided by the Division of Cancer Cause and Prevention, NCI), and the immune precipitates were collected and washed as described previously (31) by using protein A-Sepharose (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The precipitates were removed from the beads by boiling in sample buffer (1.6% sodium dodecyl sulfate, 0.01 M Tris, pH 6.8, 15% glycerol, 2% mercaptoethanol, 0.002% bromphenol blue). They were then analyzed by electrophoresis on 5 to 15% gradient polyacrylamide gels (9) and visualized by fluorography (4).

The specificity of the anti-p15 serum was tested as follows. Cells that produced an *Akv* type of MuLV (isolate WN1802B) were labeled overnight with [^{3}H]leucine and [^{3}H]lysine. Virus was partially purified from the supernatant of this culture by pelleting through 10% sucrose. The pellet was then dissolved in sample buffer. A sample of this preparation was precipitated with the anti-p15 serum and analyzed on a 7 to 17% gradient gel; only one protein, whose mobility indicated an apparent molecular weight of 15,000, was present in this immune precipitate.

Radioimmunoassays. Competition radioimmunoassays were performed by double-antibody precipitation, as previously described (28). The presence of viral gp70 was determined by using iodinated Friend MuLV gp70 and goat anti-AKR MuLV serum obtained from the Division of Cancer Cause and Prevention, NCI. Viral p30 was detected by using iodinated Rauscher MuLV p30 and goat anti-AKR p30 obtained from the Division of Cancer Cause and Prevention, NCI. The presence of viral p12 was determined by using iodinated AKR MuLV p12 and a rabbit antiserum to AKR p12 that was a kind gift from James Ihle, Frederick Cancer Research Center. The level of viral protein expression was calculated on the basis of the amount of total cellular protein (11) and the sensitivity of each assay and is expressed as nanograms of viral protein per milligram of total cellular protein.

RESULTS

Transmission of the defective MuLV genome to mink cells. In a previous report, we described the isolation of a clone of 3T3FL mouse cells, (designated AK24) which contained the genome of the defective MuLV produced by AKRSL2 cells (21). Although this genome was readily detectable in this clone by an infectivity assay for defective ecotropic MuLV's, we noted that no virus-like structures were evident in electron micrographs of these cells. We wished to develop a restriction map of the viral genome and also to determine whether this genome directed the synthesis of any known MuLV-specific proteins. However, the presence of other, endogenous MuLV genomes in the 3T3FL host cells greatly complicated these studies; therefore, we attempted to transmit the defective MuLV to nonmurine cells. The defective MuLV was rescued from clone AK24 cells by superinfection with amphotropic MuLV, as described previously (23). Mink cells were infected with the resulting virus stock at a multiplicity of infection of 0.2 complementation plaque-forming units per cell, and the cells were cloned on the following day. Individual clones were picked and screened for the presence of defective ecotropic MuLV by cocultivation with cells that produced a helper virus, as described previously (21); two clones, designated MAK26 and MAK71, were positive in these preliminary tests. These clones were then superinfected with a series of nondefective, XC-negative viruses. Infectivity assays of the viruses produced by these superinfected cells (Table 1) demonstrated that these two clones were indeed nonproducer clones since, like clone AK24, they contained a rescuable, defective ecotropic virus which registered in the complementation plaque assay.

Restriction mapping of the integrated MuLV genome in clones MAK26 and MAK71. To compare the defective MuLV genome with the genome of *Akv*, we isolated high-molecular-weight cellular DNAs from clones MAK26 and MAK71, from mink(*Akv*) cells, from AKR2B mouse cells, which also contain the *Akv* genome, and from uninfected mink cells. Hirt supernatant DNA from cells that were acutely infected with *Akv* was also prepared (20). These DNAs were then digested with restriction endonucleases, subjected to agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized with a ^{32}P-labeled probe that was representative of the full *Akv* genome (*Pst*I 8.2-kbp clone 623 DNA). Figure 1A, lanes a through f show the results obtained by *Pst*I digestion of these DNAs. *Pst*I cleaves endogenous ecotropic MuLV genomes only in the long terminal repeat (LTR), so that digestion of a full-length proviral DNA molecule generated an 8.2-kbp fragment (Fig. 2). As Fig. 1A, lanes a and b show, *Pst*I digests of MAK26 and MAK71 DNAs did contain an 8.2-kbp molecule which hybridized with the MuLV probe. As expected, this fragment was also present in the DNA preparations containing the *Akv* genome (Fig. 1A, lanes c, e, and f) but not in the preparation from normal mink cells (lane d). These results suggest that the defective MuLV genome is approximately the same size as the standard *Akv* genome and that it also resembles *Akv* in containing *Pst*I sites in its LTR.

The *Akv* genome is cleaved once by *Hin*dIII at 3.0 kbp (measured from the 5' end of the provirus) and once by *Xba*I at 7.7 kbp. To test the defective MuLV genome for the presence of these cleavage sites, we digested MAK26 and MAK71 DNAs with *Hin*dIII plus *Pst*I and with *Xba*I plus *Pst*I. As Fig. 1A, lanes g and h show, *Hin*dIII-*Pst*I digestion of these DNAs yielded fragments 3.0 and 5.2 kbp long, just as was observed with *Akv* (lanes i and k). Similarly, the *Xba*I-*Pst*I digests of these DNAs contained a large fragment (Fig. 1A, lanes m and n) which comigrated with the 7.7-kbp fragment obtained from *Akv* (lanes o and q). These results indicate that the defective MuLV genome also resembles the *Akv* genome in the placement of a *Hin*dIII site and an *Xba*I site (Fig. 2).

In addition, digestion of MAK26 and MAK71 DNAs with *Eco*RI and *Pst*I gave rise to an 8.2-kbp fragment, just as was found with *Akv*; thus,

TABLE 1. Rescue of complementation plaque-forming units from clones MAK26 and MAK71

Cell line	Superinfection[a]	CPFU/ml[b]	FIU/ml[c]
MAK26	None	$<1 \times 10^0$	$<1 \times 10^0$
	Amphotropic MuLV	4×10^4	5×10^5
	Moloney clone 83	4×10^3	3×10^5
MAK71	None	$<1 \times 10^0$	$<1 \times 10^0$
	Amphotropic MuLV	8×10^4	1×10^6
	Moloney clone 83	4×10^4	6×10^5
	Feline leukemia virus	3×10^5	ND[d]
	Baboon endogenous virus	1.6×10^3	ND
Mink	None	$<1 \times 10^0$	$<1 \times 10^0$
	Amphotropic MuLV	$<1 \times 10^0$	8×10^5
	Moloney clone 83	$<1 \times 10^0$	2×10^5
	Feline leukemia virus	$<2 \times 10^0$	ND
	Baboon endogenous virus	$<2 \times 10^0$	ND

[a] Cultures of MAK26 cells, MAK71 cells, and uninfected mink cells were superinfected with amphotropic MuLV, Moloney clone 83, feline leukemia virus, or baboon endogenous virus. After 5 days the culture fluids from these preparations were assayed for replication-defective ecotropic MuLV by the complementation plaque assay and for nondefective MuLV by the S^+L^- focus assay.

[b] CPFU, Complementation plaque-forming units.

[c] FIU, S^+L^- focus-inducing units.

[d] ND, Not determined.

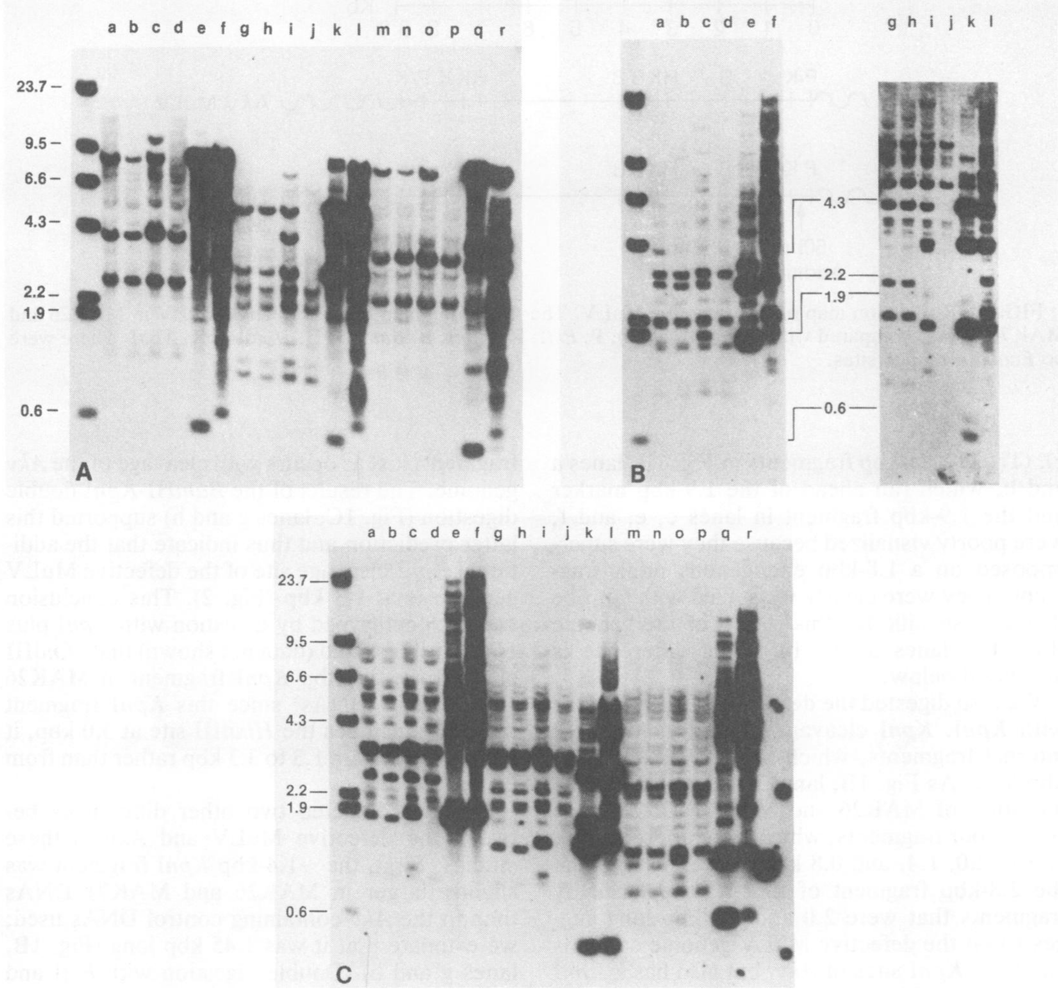

FIG. 1. Southern gel analysis of proviral DNA. DNAs were extracted, digested, fractionated by agarose gel electrophoresis, blotted onto nitrocellulose filters, and hybridized as described in the text. The DNAs used were cellular DNA of MAK26 cells (lanes a, g, and m), cellular DNA of MAK71 cells (lanes b, h, and n), cellular DNA of mink(*Akv*) cells (lanes c, i, and o), cellular DNA of uninfected mink cells (lanes d, j, and p), cellular DNA of AKR2B cells (lanes f, l, and r), and Hirt supernatant DNA from NIH/3T3 cells acutely infected with *Akv* (20) (lanes e, k, and q). (A) DNAs digested with *Pst*I (lanes a through f), *Pst*I plus *Hin*dIII (lanes g through l), and *Pst*I plus *Xba*I (lanes m through r). (B) DNAs digested with *Bam*HI (lanes a through f) and *Kpn*I (lanes g through l). (C) DNAs digested with *Bam*HI (lanes a through f), *Bam*HI plus *Kpn*I (lanes g through l), and *Bam*HI plus *Hin*dIII (lanes m through r). The DNAs were hybridized with ^{32}P-labeled *Pst*I 8.2-kbp clone 623 (A and B) or ^{32}P-labeled *Bam*HI 1.9-kbp clone 623 (C). The molecular weight markers shown at the left of each panel were a *Hin*dIII digest of ^{32}P-labeled λ DNA, and the numbers represent the lengths (in kilobase pairs) of these markers. The fragments found in uninfected mink cells represent material which hybridizes with MuLV probes and were not characterized further. Although the host range of the infectious MuLV produced by the mink(*Akv*) clone used is purely ecotropic, the restriction analysis shown here (panel A, lanes c and i; panel B, lane c; panel C, lanes c and i) indicates that more than one type of MuLV genome is actually present in these cells, as noted in the text.

*Eco*RI does not cleave the defective MuLV genome (data not shown).

We also investigated the defective MuLV genome by using digestion with *Bam*HI. This enzyme cuts *Akv* four times, yielding internal fragments that are 3.0, 1.9, and 0.4 kbp long. Digestion of MAK26 and MAK71 DNAs also produced these fragments (the 0.4-kbp fragment was inferred, since it is located between the 1.9- and 3.0-kbp fragments), except that the 1.9-kbp band migrated slightly faster (1.85 kbp) than the 1.9-kbp band from *Akv* (Fig. 1B, lanes a through

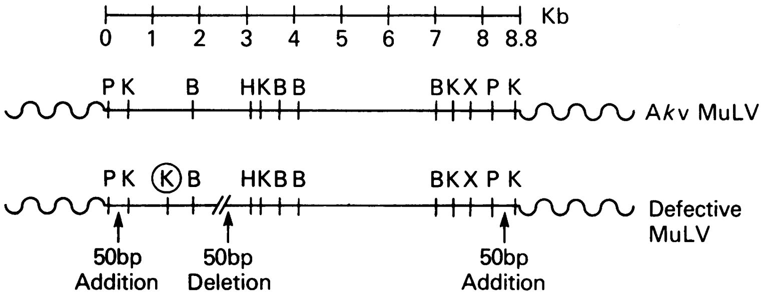

FIG. 2. Restriction map of the defective MuLV. The restriction map of the defective MuLV in MAK26 and MAK71 cells is compared with that of *Akv* (20). P, *Pst*I; K, *Kpn*I; B, *Bam*HI; H, *Hin*dIII; X, *Xba*I. There were no *Eco*RI cleavage sites.

c). (These 1.85-kbp fragments in Fig. 1B, lanes a and b, which ran ahead of the 1.9-kbp marker and the 1.9-kbp fragment in lanes c, e, and f, were poorly visualized because they were superimposed on a 1.8-kbp endogenous mink fragment. They were clearly visualized with a probe that was specific for this region of the genome [Fig. 1C, lanes a and b].) This difference is discussed below.

We also digested the defective MuLV genome with *Kpn*I. *Kpn*I cleavage of *Akv* yields three internal fragments, which are 3.9, 2.8, and 1.4 kbp long. As Fig. 1B, lanes g and h show, *Kpn*I digestion of MAK26 and MAK71 DNAs gave rise to four fragments, which were approximately 3.9, 2.0, 1.4, and 0.8 kbp long. The fact that the 2.8-kbp fragment of *Akv* was replaced by fragments that were 2.0 and 0.8 kbp long suggests that the defective MuLV genome contains all of the *Kpn*I sites of *Akv*, but also has a *Kpn*I site within the 2.8-kbp fragment. Since this additional *Kpn*I site is apparently 0.8 kbp from one end of the 2.8-kbp fragment, it is located either 1.3 or 2.5 kbp from the 5′ end of the provirus (see Fig. 2).

This additional *Kpn*I site was localized in further studies by using the 1.9-kbp *Bam*HI fragment of *Akv* as a probe. This fragment spans the region between 1.85 and 3.7 kbp in *Akv*. As Fig. 1C, lanes a and b show, this probe reacted only with the ~1.9-kbp fragment of *Bam*HI-digested MAK26 and MAK71 DNAs, as expected. If the added *Kpn*I site in these DNAs were located in the 3′ portion of the 2.8-kbp *Kpn*I fragment (i.e., at 2.5 kbp), then *Kpn*I would cleave the 1.9-kbp *Bam*HI fragment twice, yielding *Bam*HI-*Kpn*I fragments that are 0.7, 0.8, and 0.4 kbp long. On the other hand, if the additional *Kpn*I site in the defective MuLV genome were in the 5′ half of the 2.8-kbp *Kpn*I fragment (i.e., at 1.3 kbp), then *Kpn*I cleavage of the 1.9-kbp *Bam*HI fragment would generate a single large fragment that is ~1.4 kbp long plus a 0.4-kbp fragment, just as occurs with cleavage of the *Akv* genome. The results of the *Bam*HI-*Kpn*I double digestion (Fig. 1C, lanes g and h) supported this latter prediction and thus indicate that the additional *Kpn*I cleavage site of the defective MuLV genome is at 1.3 kbp (Fig. 2). This conclusion was also confirmed by digestion with *Kpn*I plus *Hin*dIII. We found (data not shown) that *Hin*dIII cleaved the 2.0-kbp *Kpn*I fragment in MAK26 and MAK71 DNAs; since this *Kpn*I fragment therefore includes the *Hin*dIII site at 3.0 kbp, it must extend from 1.3 to 3.3 kbp rather than from 0.5 to 2.5 kbp.

We also detected two other differences between the defective MuLV and *Akv* in these studies. First, the ~1.4-kbp *Kpn*I fragment was slightly larger in MAK26 and MAK71 DNAs than in the *Akv*-containing control DNAs used; we estimate that it was 1.45 kbp long (Fig. 1B, lanes g and h). Double digestion with *Pst*I and *Kpn*I localized this ~50-base-pair (bp) size difference to the 3′ side of the 3′ *Pst*I site (i.e., in the LTR) (data not shown).

Second, as noted above, the 1.9-kbp *Bam*HI fragment migrated slightly faster in MAK26 and MAK71 DNAs than in *Akv* (Fig. 1C, lanes a through c). Thus, this region of the defective MuLV genome appeared to be slightly (~50 bp) shorter than the corresponding region of *Akv*. Further information on this difference was obtained by double digestion of the cellular DNAs and hybridization with the 1.9-kbp *Bam*HI probe. As Fig. 1C shows, this difference was present in the ~1.4-kbp *Bam*HI-*Kpn*I fragment (Fig. 1C, lanes g through i) and was also present in the 1.2-kb *Bam*HI-*Hin*dIII fragment (lanes m through o). These findings show that the difference is in the region between the *Bam*HI site at 1.85 kbp and the *Hin*dIII site at 3.0 kbp in the defective MuLV genome (Fig. 2). The results shown in Fig. 1C also suggest that this region from MAK71 DNA may have been slightly shorter than the corresponding region from

MAK26 DNA, but this difference was not reproducible in other gels.

MuLV-specific proteins in MAK26 and MAK71 cells. The analysis of the defective virus genome by restriction mapping revealed three major differences between this genome and *Akv*. As discussed above, the defective genome has a slightly larger LTR, an additional *Kpn*I site at 1.3 kbp, and a small deletion between 1.8 and 3.0 kbp. We decided to determine whether any of these abnormalities in the genome was reflected in an aberrant pattern of virus-specific protein synthesis in MAK26 and MAK71 cells and whether defects in the viral proteins could account for the inability of the defective virus to replicate itself in the absence of a helper virus (21).

Initially, MAK26 and MAK71 cells were examined for the presence of MuLV-specific proteins as follows. Cultures of these cells were pulse-labeled with [^{35}S]methionine; as controls, uninfected mink cells and mink(*Akv*) cells were labeled in parallel. Cytoplasmic extracts of these cultures were then precipitated with antisera to all of the major MuLV proteins, and the labeled proteins that bound to the antisera were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Figure 3 shows the results of one such experiment, in which the four extracts were exposed to normal goat serum (Fig. 3, lanes 1 through 4), anti-p10 (lanes 5 through 7 and 9), anti-p12 (lanes 10 through 13), anti-reverse transcriptase (lanes 14 through 17), anti-p30 (lanes 18 through 21), and anti-p15 (lanes 22 through 25). This figure shows that with each of these sera, the MAK26 and MAK71 extracts (Fig. 3, lanes 2, 3, 6, 7, 11, 12, 15, 16, 19, 20, 23, and 24) were indistinguishable from the extract of uninfected mink cells (lanes 1, 5, 10, 14, 18, and 22). In contrast, Pr65^{gag} and Pr180$^{gag\text{-}pol}$ were both readily detected with all of the immune sera in the productively infected cells (lanes 9, 13, 17, 21, and 25); gPr80^{gag} and a 40,000-dalton *gag* cleavage intermediate were also visible in several of these preparations. When the MAK26 and MAK71 extracts were precipitated with anti-gp71, there was a band at 82,000 daltons (data not shown); this *env* precursor is discussed below.

The results shown in Fig. 3 suggest that the *gag* proteins and the reverse transcriptase are all completely absent from MAK26 and MAK71 cells. However, these results did not eliminate the possibility that p15 was present in these cells, since the p15 encoded by *Akv* contains no methionine and hence would not be detected in these methionine-labeled extracts (18). This seemed particularly important since p15 is the N-terminal protein in Pr65^{gag} and many defective viruses contain an N-terminal fragment of the *gag* gene product (1, 8, 19, 25, 32). Accordingly, we pulse-labeled MAK26 cells, MAK71 cells, and controls with [^{3}H]leucine and [^{3}H]lysine. Extracts of these cells were precipitated with monospecific anti-p15 serum; Fig. 4 shows gels displaying the precipitated proteins. This

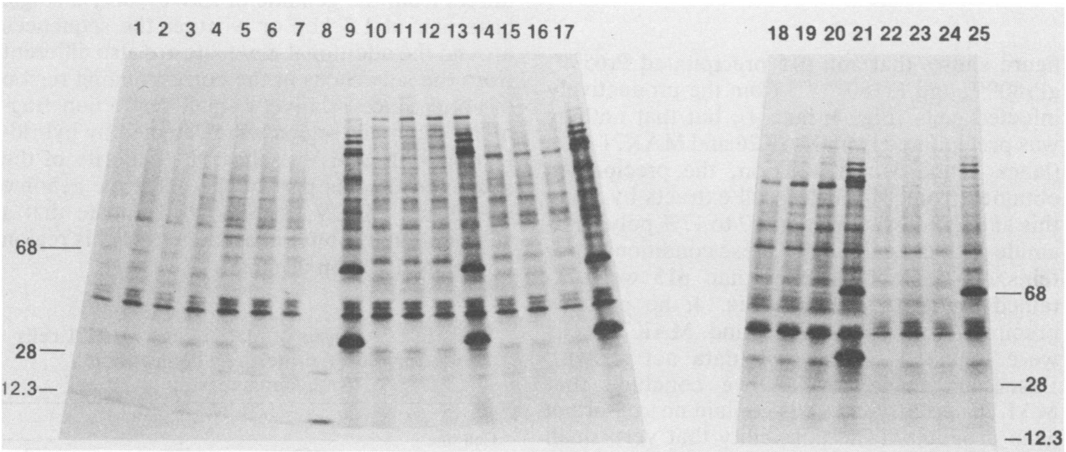

FIG. 3. Analysis of [^{35}S]methionine-labeled mink cells for MuLV-specific proteins by radioimmunoprecipitation. Cells were labeled for 30 min with L-[^{35}S]methionine (250 μCi/ml) and processed as described in the text. Extracts of mink cells (lanes 1, 5, 10, 14, 18, and 22), MAK26 cells (lanes 2, 6, 11, 15, 19, and 23), MAK71 cells (lanes 3, 7, 12, 16, 20, and 24), and mink(*Akv*) cells (lanes 4, 9, 13, 17, 21, and 25) were precipitated with normal goat serum (lanes 1 through 4), anti-p10 (lanes 5, 6, 7, and 9), anti-p12 (lanes 10 through 13), anti-reverse transcriptase (lanes 14 through 17), anti-p30 (lanes 18 through 21), and anti-p15 (lanes 22 through 25). The following molecular weight markers were used: bovine serum albumin (68,000), carbonic anhydrase (28,000), and cytochrome *c* (12,300).

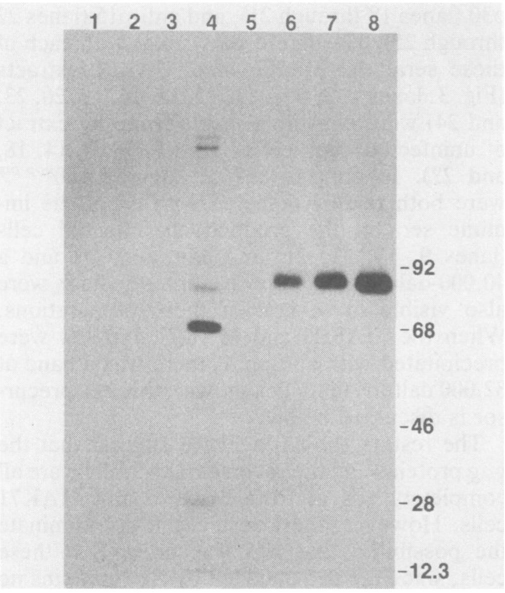

FIG. 4. Analysis of ^{3}H-labeled mink cells for MuLV-specific proteins by radioimmunoprecipitation. Cells were labeled for 30 min with L-[^{3}H]leucine and L-[^{3}H]lysine (50 μCi/ml each) and processed as described in the text. Extracts of mink cells (lanes 1 and 5), MAK26 cells (lanes 2 and 6), MAK71 cells (lanes 3 and 7), and mink(*Akv*) cells (lanes 4 and 8) were precipitated with anti-p15 (lanes 1 through 4) and anti-gp71 (lanes 5 through 8). The following molecular weight markers were used: phosphorylase *b* (92,500), bovine serum albumin (68,000), ovalbumin (46,000), carbonic anhydrase (28,000), and cytochrome *c* (12,300).

figure shows that anti-p15 precipitated $Pr65^{gag}$, $gPr80^{gag}$, and $Pr180^{gag\text{-}pol}$ from the productively infected cells (Fig. 4, lane 4), but that nothing was precipitated from MAK26 and MAK71 cells (lanes 2 and 3). In addition, the precipitates obtained from ^{3}H-labeled cell extracts by using this antiserum were run on a 7 to 17% polyacrylamide gradient gel. Under these conditions, proteins considerably smaller than p15 were retained on the gel. As in Fig. 4, no proteins precipitated from MAK26 and MAK71 cells were detected on this gel (data not shown). Based on these results, we conclude that MAK26 and MAK71 cells contain no *gag* or *pol* gene products. (The possibility that very small fragments of p15 or other *gag* proteins were present could not be excluded.)

Figure 4 also shows the results of precipitation of these ^{3}H-labeled cell extracts with anti-gp71. As shown in Fig. 4, lanes 6 and 7, a precursor was readily detected at a molecular weight of approximately 82,000. The amount and mobility of this protein were similar to the amount and mobility of the wild-type protein (Fig. 4, lane 8). Thus, MAK26 and MAK71 cells do contain the *env* gene product $gPr82^{env}$.

As an independent approach to the question of which MuLV proteins are present in MAK26 and MAK71 cells, we also tested extracts of these cells for p30, AKR-type p12, and gp71 by competition radioimmunoassays. As Table 2 shows, these cells were negative for the *gag* proteins but did contain substantial levels of proteins with gp71 determinants, as determined by these assays as well as by radioimmunoprecipitation.

DISCUSSION

Previously, we reported the isolation of a replication-defective MuLV from the AKRSL2 line of cultured AKR leukemia cells (21). In this work we analyzed the defectiveness of this virus in molecular terms and compard it with *Akv*, the endogenous ecotropic virus of AKR mice. We studied the genomes of these two MuLV's by mapping with a variety of restriction endonucleases. Three relatively small differences were found (Fig. 2). First, the defective virus contains a *Kpn*I cleavage site at 1.3 kbp from the 5′ end of the provirus, whereas this site is not found in *Akv*; second, the defective genome is approximately 50 bp shorter than *Akv* in the region between 1.8 and 3.0 kbp from the 5′ end; and third, the LTR of the defective genome is ~50 bp longer than the LTR of *Akv*.

It is not known whether the defective genome differs from the genome of *Akv* by only a single base pair at 1.3 kbp or whether the sequences around the additional *Kpn*I site are also different from the sequences in the corresponding region of *Akv*. Since relatively small restriction fragments from this region were detected by hybridization with MuLV probes, the majority of the sequences in this part of the defective genome are clearly MuLV related. We estimate that a substitution of nonviral sequences in this region could span 400 bp at most.

TABLE 2. Analysis of MAK26 and MAK71 cells for MuLV proteins by competition radioimmunoassay

Cell line	Amt of:[a]		
	p12	p30	gp71
Mink	<1	<1	<10
MAK26	<1	<1	531
MAK71	2	<1	288
Mink(*Akv*)	750	ND[b]	1,380

[a] Results are expressed as nanograms of MuLV protein per milligram of cellular protein.

[b] ND, Not determined.

As noted above, the defective MuLV could have originated from *Akv* in the somatic tissues of the mouse which gave rise to the AKRSL2 leukemia cell line, or it could be a distinct endogenous virus. The fact that the region between 1.8 and 3.0 kbp is ~50 bp shorter in the defective genome than in *Akv* shows that, if the defective genome is derived from *Akv*, then its origin must have involved deletions or recombinational events or both, not just single-base changes.

Finally, the defective genome differs from the *Akv* isolates used as controls in these experiments with respect to the length of the LTR. However, recently the endogenous ecotropic MuLV's have been found to be polymorphic in this regard. The LTR of the defective virus is the same size as the LTRs in other nondefective MuLV's (20); thus, its size is fully consistent with normal LTR functions, such as replication, integration, and transcription of the viral DNA. The fact that the defective MuLV replicates to a high titer in the presence of a helper virus (Table 1) (21) suggests that all three of these functions are performed efficiently by the defective genome. In particular, the efficient rescue observed implies that full-length, genomic RNA is present at normal levels in the cytoplasm of infected cells, whereas the production of *env* protein in nonproducer cells (Fig. 4 and Table 2) suggests that the *env* message is synthesized, processed, and translated normally.

We conclude that the defective MuLV produced by AKRSL2 cells has a restriction map which is similar but not identical to that of *Akv*. These results are consistent with the hypothesis that the defective MuLV was derived from *Akv*, but do not completely exclude the possibility that this virus is a distinct endogenous virus. Further studies will be required before the origin and significance of the defective virus can be established. (We also cannot exclude the possibility that the defective viral genome was changed by mutation or recombination in the two replicative cycles which occurred between its release from AKRSL2 cells and its analysis in MAK26 and MAK71 cells.)

We also tested cells that contained the defective MuLV for virus-specific proteins. No products of the *gag* or *pol* genes were detected in these cells (Fig. 3 and 4 and Table 2). This defect obviously accounts for the inability of the defective MuLV to replicate in the absence of a helper virus and for the absence of any particles visible by electron microscopy (21).

As discussed above, the defective MuLV genome contains a *Kpn*I cleavage site at 1.3 kbp, which is not present in nondefective *Akv*. Since the *gag* coding region is believed to begin at 1.1 kbp (C. van Beveren and I. Verma, personal communication), this cleavage site is probably in the middle of the p15 gene. One possible explanation for the absence of any protein that was precipitable with our anti-p15 serum is that the abnormal nucleotide sequence at 1.3 kbp introduces a frameshift or termination codon into the gene. In this case a protein containing an N-terminal portion of p15 would be synthesized. Our failure to detect this protein might be due to its small size or instability; it is also possible that this p15 fragment lacks antigenic determinants recognized by our antiserum. Alternatively, the defective genome could also be altered upstream from 1.3 kbp, so that *gag* translation is completely prevented by a frameshift or termination codon or by some other alteration.

Although cells containing the defective MuLV appear to lack *gag* and *pol* proteins, they clearly contain an *env* gene product. The presence of an *env* gene product was not unexpected, since cells containing both the defective MuLV and an XC-negative helper virus fuse XC cells (21). Several lines of evidence (23, 30, 33) suggest that XC cell fusion is a property of ecotropic *env* molecules; our present finding that the defective virus that exhibits this phenotype apparently codes only for the *env* protein constitutes strong additional support for this conclusion. (The requirement for coinfection with a nondefective helper virus is not yet understood). We also observed that when the defective MuLV was rescued by a helper virus which normally is not able to infect mouse cells, such as feline leukemia virus or baboon endogenous virus, the resulting pseudotype particles registered in the standard complementation plaque assay on mouse cells (Table 1). It seems likely that the defective genome encodes a fully functional *env* protein, which can be incorporated into virions and gives virions the ability to infect mouse cells. Further analysis of this protein is now underway.

Besmer et al. (3) have described a mutant of Moloney MuLV which synthesizes an *env* protein and a 45,000-dalton fragment of the *gag* gene product. To our knowledge, however, this is the first description of a nontransforming mammalian retrovirus genome which synthesizes a complete *env* protein but no detectable *gag* or *pol* gene product. It should be noted that a defective virus of this type would not be detected by most virus assays, since it does not produce virus particles, has no DNA polymerase activity, and is negative in sensitive immunological tests for the viral core proteins (Table 2). Cells containing this type of defective virus should be useful reagents for a variety of genetic experiments. In addition, they should provide a unique opportunity to analyze the processing and subcellular localization of the *env* protein in the absence of *gag* proteins and thus to determine the role, if any, of *gag* proteins in *env* maturation.

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