Cells nonproductively transformed by Abelson murine leukemia virus express a high molecular weight polyprotein containing structural and nonstructural components

(RNA tumor viruses/transforming proteins/*gag* gene-coded translational products/pseudotype virions/ virus-coded phosphoproteins)

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ABSTRACT   Cell clones nonproductively transformed by the replication-defective Abelson strain of murine leukemia virus (AbLV) were analyzed for type C viral antigen expression by competition immunoassay. AbLV-transformed mink non-producer lines were found to express a 110,000- to 130,000-molecular weight polyprotein containing murine leukemia virus *gag* proteins p15 and p12 covalently linked to nonstructural AbLV-coded component(s) of around 80,000–100,000 molecular weight. This polyprotein lacked detectable antigenic cross-reactivity with other virion-coded *gag* gene proteins such as p30, p10, the viral reverse transcriptase (RNA-dependent DNA polymerase), or the major viral envelope glycoprotein, gp70. By analogy to earlier data on feline and avian sarcoma viruses, these results suggest that a portion of this polyprotein might represent the AbLV *src* gene product and that in translation it is initially linked in precursor form to *gag* structural proteins. Superinfection of mink cells nonproductively transformed by AbLV—with either a wild mouse amphotropic type C virus isolate, 4070-A, or with the endogenous cat virus, RD114—led to production of pseudotype virus containing high concentrations of the AbLV-coded precursor polyprotein.

Mammalian type C RNA tumor viruses exist as either infectious leukemia viruses or as replication-defective transforming viruses. The infectious viruses replicate in, but do not morphologically transform, fibroblast embryo cultures (1) and exist in a naturally integrated state in the cellular genome of a broad range of mammalian species (2, 3). In contrast, transforming viruses frequently cause morphologic alteration of fibroblasts in cell culture (1), require helper viruses for completion of their replication cycle (1, 4), and cause fibrosarcoma (5, 6), lymphoma (7), or erythroleukemia (8) upon *in vivo* inoculation into appropriate hosts. Such transforming viruses appear to have arisen as a result of genetic recombination between type C leukemia viruses and host cell genetic sequences coding for malignant transformation (9, 10).

The type C viral genome contains discrete regions that code for the major nonglycosylated virus structural proteins (*gag*), the reverse transcriptase (RNA-dependent DNA polymerase) (*pol*), and the envelope glycoprotein (*env*) (11). On the basis of indirect evidence (12, 13) and by analogy to avian type C viruses (14), the order of these genes within the mammalian type C viral genome appears to be 5'-*gag-pol-env*-3'. The primary translational product of the *gag* gene has been shown to be a 65,000-molecular weight (M_r) precursor that, upon post-translational processing, gives rise to structural proteins of 30,000- (p30), 15,000- (p15), 12,000- (p12), and 10,000- (p10) M_r (15, 16). The internal arrangement of the *gag* gene has been

established as 5'-p15-p12-p30-p10-3' for both murine (16, 17) and feline (18) type C virus isolates. Replication-defective sarcoma viruses contain an additional transforming gene designated *src*, apparently derived from the host from which the virus was originally isolated (10, 19). Cells nonproductively transformed by each of several different replication-defective transforming viruses have been shown to express differing numbers of helper virus structural proteins (16, 18, 20). With one possible exception (21), such expression appears to be limited to the *gag* gene-coded proteins and occurs in a progressive manner from the 5' to 3' terminus of the *gag* gene (16, 18, 20, 22).

Mammalian cells nonproductively transformed by feline sarcoma virus (FeSV) express a 130,000-M_r polyprotein that contains antigenic determinants in common with the two FeLV amino terminal *gag* gene proteins, p15 and p12 (18, 23), and also crossreacts with the tumor-specific feline oncornavirus-associated cell membrane antigen (FOCMA) (18, 23). Post-translational cleavage of this protein gives rise to a 60,000-M_r component that contains FOCMA crossreactive determinants and a 25,000-M_r component containing p15 and p12 (23). In the present study, cell clones nonproductively transformed by the replication-defective Abelson strain of murine leukemia virus (AbLV) were analyzed for type C viral antigen expression. The results indicate the presence of an 110,000- to 130,000-M_r polyprotein containing p15 and p12 antigenic determinants covalently linked to nonstructural components of around 100,000 M_r that may include AbLV-coded transforming sequences.

MATERIALS AND METHODS

Cells and Virus. Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Company, Denver, CO). Cell lines included NIH/3T3 mouse cells nonproductively transformed by Abelson murine leukemia virus (ANN-1) (24); a fetal lung mink cell line, CCL 64, obtained from the American Type Culture Collection, Rockville, MD; and a subclone of CCL 64, designated 64 M1, nonproductively transformed by the Moloney strain of murine sarcoma virus, kindly provided by G. J. Todaro, National Cancer Institute. CCL 64 mink clones nonproductively transformed by AbLV will be described elsewhere. Type C RNA viruses included the Moloney (M-), AKR-, and Rauscher (R-)

Abbreviations: M_r, molecular weight; AbLV, Abelson strain of murine leukemia virus; MuLV, murine leukemia virus; M-MuLV and R-MuLV, Moloney and Rauscher strains of MuLV; FeSV, feline sarcoma virus; FeLV, feline leukemia virus; FOCMA, feline oncornavirus-associated cell membrane antigen.

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strains of murine leukemia virus (MuLV), an amphotropic wild mouse virus, 4070-A (25), as well as two endogenous xenotropic viruses of mouse cells, BALB:virus-2 and NIH-MuLV (26). An endogenous type C virus isolate of feline cells, RD114 (27), was also used. Viruses were obtained as sucrose gradient-purified preparations obtained through the courtesy of R. V. Gilden (Frederick Cancer Research Center).

Double Antibody Competition Immunoassays. Competition immunoassays for type C virus structural proteins including M-MuLV p12, AKR-MuLV p15, R-MuLV p30, R-MuLV p10 (28, 29), M-MuLV gp70 (unpublished), and R-MuLV reverse transcriptase (30) were performed according to previously described methods.

RESULTS

AbLV-Transformed Nonproducer Cell Clones Express MuLV p12 and p15 in the Absence of Other MuLV *gag, pol,* or *env* Gene-Coded Translational Products.

As a preliminary step in a determination of the nature of the replication-defective AbLV genome, the original AbLV-transformed mouse cell line, ANN-1, and each of five AbLV-transformed mink nonproducer lines were subjected to immunologic analysis. Each was tested in competition immunoassays for translational products of the type C viral genes, *gag, pol,* and *env.* As summarized in Table 1, ANN-1 was found to express the two amino-terminal MuLV *gag* gene-coded proteins, p15 and p12, at relatively high level. While the remaining *gag* gene-coded proteins, p30 and p10, and the *env* gene product, gp70, were also detectable, the levels of expression of these proteins were no greater than those observed in extracts of the nontransformed control mouse cell line, NIH/3T3. These findings suggest that the replication-defective AbLV genome codes for two MuLV *gag* gene products, p15 and p12. The detection of low levels of other type C viral proteins in control NIH/3T3 cells is consistent with our previous demonstration of endogenous xenotropic virus expression in embryo cell lines derived from most inbred strains of mice (26, 31). Further evidence that the AbLV genome codes for the amino-terminal portion of the MuLV *gag* gene was obtained by the demonstration of MuLV p12 and p15 expression in each of five AbLV-transformed nonproducer mink clones in the absence of significant reactivity in competition immunoassays for p30, p10, or gp70 (Table 1).

To test for reverse transcriptase expression in the above AbLV-transformed nonproducer clones, broadly reactive and highly sensitive competition immunoassay was developed, utilizing antibody directed against detergent-disrupted M-MuLV for precipitation of ^{125}I-labeled R-MuLV reverse transcriptase. By use of this assay, reverse transcriptases of a wide
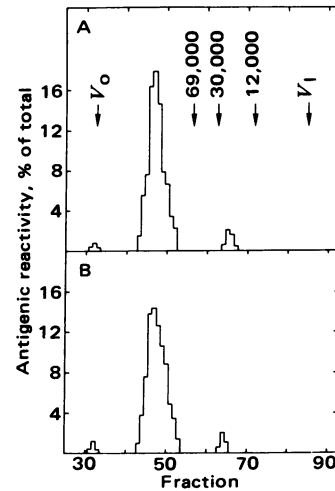

FIG. 1.   Molecular size analysis under nondenaturing conditions of MuLV *gag* gene-coded proteins expressed in a representative AbLV-transformed mink nonproducer clone, 64Ab2. Cell extract (20 mg) was subjected to acrylamide/agarose gel filtration (Ultrogel AcA .34) according to previously described methods (28), and individual column fractions were tested in heterologous competition immunoassays utilizing antibody to detergent-disrupted R-MuLV for precipitation of either ^{125}I-labeled M-MuLV p12 (A) or ^{125}I-labeled M-MuLV p15 (B). Results are expressed as the percentage of total antigenic reactivity in each column fraction and are based on the degree of displacement of competition curves relative to standards. Molecular weight markers included tracer amounts of ^{125}I-labeled bovine serum albumin (69,000), R-MuLV p30 (30,000), R-MuLV p12 (12,000), and free iodide (V_I). V_o, void volume.

range of type C virus isolates of mouse origin were found to react efficiently and to similar final extents (data not shown). As summarized in Table 1, each of the AbLV-transformed clones analyzed was found to lack detectable levels of antigenic reactivity despite the fact that the reverse transcriptase competition immunoassay was equally as sensitive as assays for MuLV *gag* gene translational products.

MuLV *gag* Gene-Coded Proteins Expressed in AbLV-Transformed Nonproducer Cells Are in the Form of High-M_r Precursors. To further characterize the MuLV-crossreactive antigenic reactivities in AbLV-transformed nonproducer cells, extracts were subjected to molecular size analysis, and individual column fractions were tested for MuLV p15 and p12. In the case of the AbLV-transformed mink cell line 64Ab2, when subjected to nondenaturing gel filtration, over 90% of both p15 and p12 reactivities cochromatographed at a molecular weight of about 110,000–130,000 with a small amount of reactivity for both proteins at 25,000 M_r (Fig. 1). The possibility

Table 1.   Expression of the type C virus structural protein in cells nonproductively transformed by Abelson leukemia virus

Cell line	Designation	Viral protein expression, ng viral protein/mg total cellular protein*					
		p15	p12	p30	p10	RDDP	gp70
Normal mouse	NIH/3T3	30	25	50	20	<5	35
AbLV-transformed mouse	ANN-1	1830	2160	82	30	<10	30
M-MuLV-infected mouse	M-NIH/3T3	4610	3150	8320	2950	60	5200
Normal mink	CCL64	<5	<5	<5	<5	<5	<20
AbLV-transformed mink	64Ab2	2530	1770	<5	<5	<5	<20
AbLV-transformed mink	64Ab4	2150	1470	<5	<5	<5	<20
AbLV-transformed mink	64Ab7	2280	1220	<5	<5	<5	<20
AbLV-transformed mink	64Ab8	2500	1650	<5	<5	<5	<20
AbLV-transformed mink	64Ab9	1640	1200	<5	<5	<5	<20

* Cellular extracts were tested at serial 2-fold dilutions in heterologous immunoassays utilizing goat antisera against detergent-disrupted M-MuLV for precipitation of ^{125}I-labeled R-MuLV p15, p12, p30, p10, reverse transcriptase (RDDP), and gp70. The results are based on extent of displacement relative to known protein standards and represent mean values from three separate determinations.
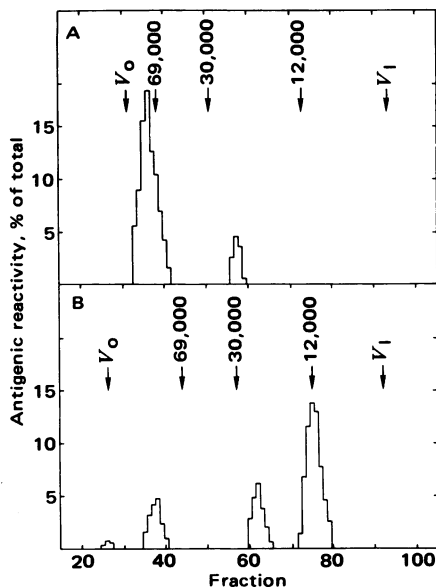
FIG. 2. Molecular size analysis under denaturing conditions of MuLV *gag* gene proteins expressed in AbLV-transformed nonproducer mink 64Ab2 (*A*) and mouse ANN-1 (*B*) cell clones. Cell extracts (20 mg) were subjected to agarose gel filtration (Bio-Gel A-15m) in the presence of 6 M guanidine-HCl (17), and individual column fractions were tested in a heterologous competition immunoassay using antibody to R-MuLV for precipitation of ^{125}I-labeled M-MuLV p12 as described for Fig. 1.

that the high-M_r p15, p12 antigenic reactivity might represent an aggregate was ruled out by the fact that a similar peak of reactivity was observed when the clone 64Ab2 extract was subjected to gel filtration in the presence of 6 M guanidine-HCl (Fig. 2*A*). Analysis of two additional AbLV-transformed mink cell clones yielded similar 110,000- to 130,000-M_r peaks of antigenic reactivity (data not shown). In contrast, when subjected to similar analysis under either nondenaturing (data not shown) or denaturing (Fig. 2*B*) conditions, ANN-1 cells were found to express the 110,000- to 130,000-M_r polyprotein at much lower concentration. Proportionately higher peaks of p12 antigenic reactivity were observed at molecular weights of 25,000 and 12,000 (Fig. 2*B*). While a low level of antigenic reactivity corresponding to MuLV p15 was located at 110,000–130,000 M_r, most such reactivity chromatographed as peaks at molecular weights of 25,000 and 15,000 (data not shown).

As an independent test of the specificity of the MuLV *gag* gene-coded immunologic reactivities in the ANN-1 and 64Ab2 clones, extracts of each were tested in a series of highly type-specific p12 immunoassays. As shown in Fig. 3, cell extracts prepared from both the ANN-1 and 64Ab2 clones competed equally as well (>90%) as density-gradient-purified M-MuLV in a homologous immunoassay for M-MuLV p12. In contrast, each of a number of other prototype mouse type C viruses tested, including AKR-MuLV, BALB:virus-2, R-MuLV, and NIH-MuLV, competed to final extents of only around 50%. Moreover, neither the ANN-1 nor the 64Ab2 extracts competed to more than 50% in homologous competition immunoassays for AKR-MuLV, BALB:virus-2, R-MuLV, or NIH-MuLV p12 (data not shown). These findings are consistent with the fact that the helper virus in the stock from which AbLV was originally derived was M-MuLV (16) and further establish the specificity of the p12 reactivity in AbLV-transformed nonproducer cell lines.

Isoelectric Points of M-MuLV p15- and p12-Containing Polyproteins Expressed in AbLV-Transformed Nonproducer Cells. Partially purified polyproteins from 64Ab2 cells were
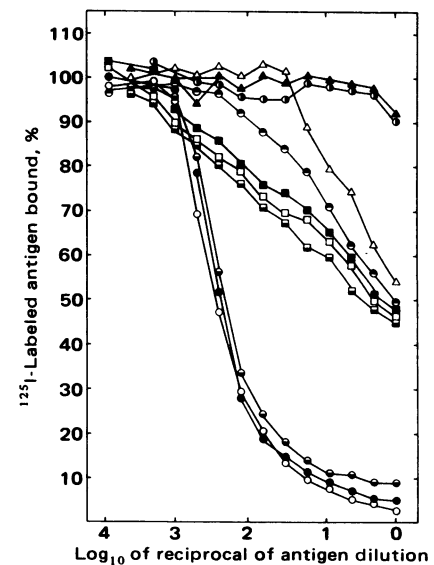

FIG. 3. Homologous competition immunoassay for the highly type specific 12,000-M_r *gag* gene-coded protein (p12) of M-MuLV. Unlabeled detergent-disrupted viruses (50 μg/ml) and cellular extracts (3 mg/ml) were tested at serial 2-fold dilutions for ability to compete with ^{125}I-labeled M-MuLV p12 for binding limiting amounts of antibody to detergent-disrupted M-MuLV. Results are expressed as mean values from two separate determinations and are normalized to 100% maximal binding at infinite antigen dilution. Viruses tested as competing antigens included AKR-MuLV (■), NIH-MuLV (⊖), BALB:virus-2 (□), R-MuLV (▨), and M-MuLV (○). Cell extracts included: ANN-1 (⊙), NIH/3T3 (▲), 64Ab2 (●), CCL64 (◍), and 64 M1 (△).

subjected to preparative isoelectric focusing, and individual fractions were analyzed by competition immunoassay for both M-MuLV p15 and p12 reactivity. For comparison, density-gradient-purified M-MuLV was subjected to similar analysis. As shown in Fig. 4, the isoelectric point of the 25,000-M_r precursor was 5.8, and thus intermediate between values obtained for M-MuLV p15 (pI 7.6) and p12 (pI 4.5). In contrast, the 110,000- to 130,000-M_r polyprotein isolated from the AbLV-transformed mink line 64Ab2 focused at a somewhat lower isoelectric point (pI 4.2), indicating that the nonstructural protein component of this AbLV-coded polyprotein is very acidic. The degree of acidity points to a composition for the 100,000-M_r component of the polyprotein uniquely different from the viral structural proteins.

AbLV(RD114) Pseudotype Virions Incorporate the 130,000-M_r AbLV-Coded Polyprotein in an Uncleaved Form. Previously an FeSV-coded high-M_r precursor polyprotein containing feline leukemia virus (FeLV) p15 and p12 covalently bound to a nonstructural component of around 100,000 M_r (23) was shown to be incorporated into pseudotype virions in an uncleaved form (33, 34). It was of interest to attempt by a similar approach to rescue and partially purify the 110,000- to 130,000-M_r polyprotein described in the present study. For this purpose, cultures of the AbLV-transformed mink clone 64Ab2 were superinfected with an endogenous feline virus, RD114, or the wild mouse amphotropic isolate 4070-A. Following purification by density gradient centrifugation, the resulting pseudotype virions were subjected to molecular sizing analysis under denaturing conditions, and column fractions were analyzed by competition immunoassay. As shown in Fig. 5*A*, AbLV(RD114) pseudotype virus contained immunologic reactivity in the M-MuLV p12 assay chromatographing at a relatively high molecular weight (110,000–130,000), while ad-
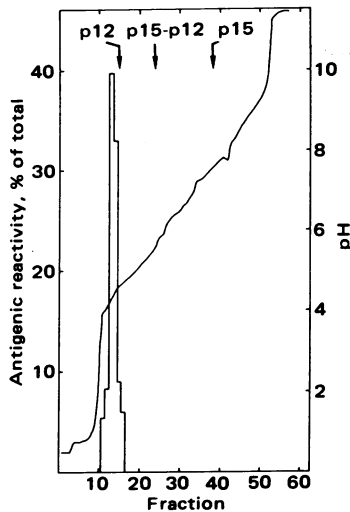
FIG. 4. Isoelectric focusing analysis of the 110,000- to 130,000-M_r precursors partially purified from the AbLV-transformed mink nonproducer cell clone 64Ab2. Antigen-positive fractions from the 100,000- to 130,000-M_r region of the AcA 34 gel filtration column described for Fig. 1 were pooled, dialyzed at 4° against 0.01 M Tris·HCl (pH 7.8), lyophilized, resuspended in 1 ml of water, and subjected to isoelectric focusing (32). Two-milliliter fractions were collected and analyzed by competition immunoassay utilizing antibody to M-MuLV for precipitation of ^{125}I-labeled M-MuLV p12. Results are expressed as the percentage of total antigenic reactivity in each column fraction as described for Fig. 1. The isoelectric points for M-MuLV p12 and p15 and for the p15-p12 precursor, indicated by vertical arrows, were determined on separate columns.

ditional less pronounced peaks of reactivity were observed at 25,000 and 12,000 M_r. Both the 110,000- to 130,000-M_r and 25,000-M_r peaks were also found to contain antigenic reactivity for M-MuLV p15 (data not shown). In contrast, similar analysis of AbLV(4070-A) pseudotype virus released a major peak of M-MuLV p12 at around 70,000 M_r with additional peaks of reactivity at 25,000 and 12,000 M_r. Interestingly, column fractions containing M-MuLV p12 reactivity at 70,000 M_r were not detectably reactive in the M-MuLV p15 assay (data not shown). These findings establish that the 110,000- to 130,000-M_r polyprotein coded for by the AbLV genome as well as several intermediate cleavage proteins are efficiently incorporated into pseudotype viruses.

DISCUSSION

AbLV is a replication-defective transforming virus, derived by *in vivo* passage of M-MuLV in mice (7), and appears to represent a genetic recombinant between M-MuLV and host cellular sequences responsible for malignant transformation (35). Upon inoculation *in vivo*, AbLV induces B-cell lymphoid leukemia, while *in vitro* the virus has been shown to transform both lymphoid cells (36) and embryo fibroblasts (24). Due to the previous demonstration that cells nonproductively transformed by AbLV express the amino-terminal portion of the MuLV *gag* gene (16), it was reasoned that the recombinational event leading to acquisition of transforming sequences may have occurred within the region of the genome corresponding to *gag* and that the transforming protein might be synthesized in the form of a precursor containing a portion of the MuLV *gag* gene product. The results obtained indicate that p15 and p12 are initially synthesized in the form of a 110,000- to 130,000-M_r precursor polyprotein that is cleaved, producing initially a 25,000-M_r precursor containing p15 and p12 and subsequently p15 and p12 at molecular weights of 15,000 and 12,000, re-
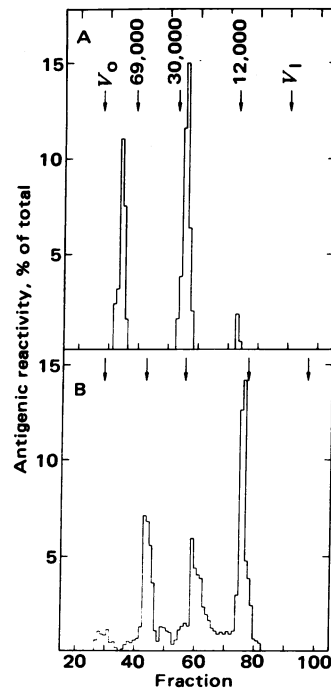

FIG. 5. Molecular size analysis of AbLV *gag* gene-coded proteins incorporated in pseudotype virions using either RD114 (*A*) or wild mouse amphotropic 4070-A (*B*) as helper virus. Sucrose-density-gradient-purified virus (10 mg) was subjected to agarose gel filtration in the presence of 6 M guanidine·HCl according to previously described methods (17). One-milliliter fractions were collected and analyzed in a homologous M-MuLV p12 competition immunoassay as described in the legend to Fig. 4. ^{125}I-Labeled M_r markers were as in Fig. 1.

spectively. The virus-coded nature of the entire 110,000- to 130,000-M_r polyprotein is established by its efficient translation in *Xenopus laevis* oocytes using AbLV genomic RNA as message (37).

Awaiting resolution is the question of whether the nonstructural component of the AbLV-coded polyprotein is located at its amino or the carboxy terminus. If it occupies a carboxy-terminal position, as would be predicted by the above model, the 110,000- to 130,000-M_r precursor probably contains a small portion of p30 and thus the p12-p30 cleavage site (38). This would account for the post-translational processing of the 110,000- to 130,000-M_r precursor and is consistent with the lack of detectable p30 or p10 in the AbLV nonproducer cells, because the expression of a small portion of the p30 molecule would not be detected by the immunologic assays used in the present study.

Several identities are possible for the nonstructural component of the high-M_r polyprotein expressed in AbLV-transformed cells. Although the present findings argue against the possibility that it is an immunologically reactive translation product of either *env* or *pol* genes, the possibility that it contains immunologically nonreactive sequences coded for by either *pol* or *env* cannot be excluded. Alternatively, it might represent a nonsense protein produced as a result of a small deletion causing a frame shift in translation of a portion of the *gag* and *pol* genes. This possibility, however, seems unlikely in view of the high probability of encountering a termination codon prior to completion of translation. Finally, the nonstructural component of the AbLV-coded polyprotein could represent the translational product of the AbLV *src* gene or the product of another cellular gene also linked to the viral structural genetic information by a recombination event. The weight of indirect

evidence would seem to favor its identity as a *src* gene product, on the basis of analogy to findings recently obtained in studies of other RNA tumor viruses. For instance, in the feline system, a 110,000- to 130,000-M_r translational product of the FeSV genome containing FeLV p15 and p12 linked to a 60,000-M_r nonstructural component has been shown to react with tumor-specific antisera (23, 33, 34). An avian RNA tumor virus-(MC29) coded polyprotein consisting of two avian leukemia virus *gag* gene proteins, p19 and p27, linked to a 60,000-M_r nonstructural protein has also been described (39). By oligonucleotide map analysis, the region of the viral genome coding for this latter protein has been shown to be absent from the leukemia virus genome from which MC29 was derived (40).

In view of both the present findings and our previous results with the FeSV genome (18, 23), the extent of post-translational processing of sarcoma virus-coded p15-p12-containing precursors appears influenced by the species of origin of the non-productively transformed cell and by the helper virus used in the production of pseudotypes. For instance, while neither the AbLV-coded (present findings) nor the FeSV-coded (23) polyprotein precursors are cleaved to more than limited extents in mink nonproducer lines, they undergo extensive cleavage as nonproductive transformants of mouse or rat cells, respectively. Moreover, when incorporated into virions, the FeSV-coded 110,000- to 130,000-M_r precursor is fully cleaved when FeLV is used as helper virus but not when either Kirsten MuLV or RD114 is utilized (34). Similarly, the present findings demonstrate the incorporation of the uncleaved 110,000- to 130,000-M_r AbLV-coded polyprotein in AbLV(RD114) pseudotype virions. In contrast, the major AbLV-coded protein observed using the amphotropic helper virus 4070-A for rescue of AbLV was a 70,000-M_r intermediate cleavage product containing p12 in the absence of detectable p15 reactivity. These findings have important practical implications because by choosing both the appropriate helper virus and cell line for virus propagation it should be possible to obtain a relatively large yield of the uncleaved AbLV-coded 130,000-M_r polyprotein. By similar means it may be possible to purify AbLV-coded translational products and develop sensitive immunologic approaches for exploring the possible expression of an immunologically crossreactive antigen in appropriate animal tumor systems. In fact, in the feline system this approach has recently led to the successful isolation of the 130,000-M_r FeSV-coded protein (34).

Note Added in Proof. Witte *et al.* (41) have recently reported a 120,000-M_r AbLV-coded polyprotein analogous to the 110,000- to 130,000-M_r precursor polyprotein described in the present study.

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