Leukemogenesis by Gross passage A murine leukemia virus: Expression of viruses with recombinant env genes in transformed cells

(intrathymic injection/primary thymoma/virus isolation/primary env gene product/peptide mapping)

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ABSTRACT Gross passage A murine leukemia virus (MuLV) derived from extracts of C3Hf/Bi mouse leukemias has been shown to be a virus complex consisting of ecotropic, xenotropic, and recombinant, dualtropic MuLV components. The three virus components were distinguished biochemically by differences in the molecular weights and peptide maps of their primary env gene products synthesized in infected cells in vivo and in vitro. Virus expression was studied in primary leukemias induced in C3Hf/Bi mice by Gross passage A virus extracts and by the individual ecotropic and recombinant MuLV components that were isolated in vitro. Our findings suggest that expression of the recombinant MuLV component of the Gross passage A virus complex is necessary and sufficient for the induction of leukemias in C3Hf/Bi mice. In contrast, induction of leukemias by the ecotropic virus component appears to involve generation of a second virus with characteristics of recombinant, dualtropic MuLV.

Considerable recent evidence indicates that genetic recombination between the env genes of ecotropic- and xenotropic-related murine leukemia virus (MuLV) is essential for acquisition of viral determinants of leukemogenicity in mice (1–16). Apparently inconsistent with this concept are results with Gross passage A virus, originally derived from extracts of a spontaneous AKR mouse leukemia by serial passage in mice of the low leukemia incidence strain C3Hf/Bi (17). Only ecotropic MuLV has been isolated in vitro from Gross passage A extracts of C3Hf/Bi leukemias (2, 18, 19). The cloned Gross ecotropic MuLV (G-MuLV) induces leukemias in C3Hf/Bi mice, although with a longer latency period and lower incidence than the original Gross passage A extracts (19, 20). The difference in biological activity between mouse-passaged virus and the virus isolated and propagated in tissue culture has not been explained. One possibility that we have examined in this study is that the difference in biological activity between in vivo and in vitro passaged Gross virus is due to loss of a hitherto undetected virus component during isolation procedures in vitro. Using a protocol that can detect and distinguish biochemically ecotropic, xenotropic, and recombinant, dualtropic MuLV components existing as virus mixtures (21), we have determined that Gross passage A virus is a mixture consisting predominantly of ecotropic and recombinant MuLV components. The isolated recombinant MuLV alone is leukemogenic. The isolated ecotropic MuLV is leukemogenic also but this is apparently due to its ability to reaggregate a recombinant MuLV component in vitro.

MATERIALS AND METHODS

Mice. C3Hf/Bi mice were obtained from the Core Breeding Facility of this institute. This strain of mice has been maintained by brother–sister mating in our colony from founder stock obtained in 1961 by L. J. Old and E. A. Boyse from L. Gross. All mice used in our experiments were immunized against Sendai virus.

Viruses. Passage 104 of Gross passage A virus was provided generously by Ludwik Gross. Gross passage A virus is defined as a 10% (wt/vol) extract in physiological saline of target tissues (thymus and mesenteric lymph node) of a C3Hf/Bi mouse leukemia (17). Subsequent passages in C3Hf/Bi mice and preparation of cell-free extracts were performed as described by Gross et al. (17, 20) with the exception that extracts were filtered through 0.8-μm Millex filters (Millipore Corp.) prior to use. Pool 183B1 of the tissue culture virus (G-MuLV) isolated from Gross passage A virus was obtained from Janet W. Hartley. The tissue culture history of this virus isolate has been described (19). Isolation and characterization of cloned AKR ecotropic, xenotropic, and dualtropic [mink cell focus-inducing (MCF)] MuLV 69E5, 69X9, and 69L1, respectively, have been described (14).

Virus isolation in vitro and Bioassay. Virus recovery in vitro was performed by infection of either mouse SC-1 cells or mink CCL64 cells with 0.5 ml of cell-free virus extract according to standard procedures. Infected cells were subcultured at 7-day intervals and assayed for expression of MuLV gag-coded p30 antigen at each subculture by indirect immunofluorescence of acetone-fixed cells using a rabbit antiserum to p30 protein of Rauscher (R)-MuLV (14). All cultures of infected cells used in pulse-labeling experiments were 100% positive for expression of p30 antigens.

Suckling mice (3–5 days of age) were injected intraperitoneally (i.p.) with 0.1 ml of virus inoculum and weaned at approximately 21 days of age. All mothers of virus-injected mice were sacrificed at weaning. Weanling mice were injected by the intrathymic (i.t.) route with 0.15 ml of virus inoculum as described (22). Virus inocula consisted of filtered cell-free extracts of thymomas or supernatants of chronically infected tissue culture cells. Control mice were injected with either phosphate-buffered saline (extracts) or Dulbecco’s modified Eagle’s minimal essential medium supplemented with 10% heat-inactivated fetal calf serum (culture supernatants).

Radiolabeling and Immunoprecipitation of Virus-Infected Fibroblasts and Primary Leukemia Cells. Petri dishes (100 mm) of confluent virus-infected fibroblasts were pulse-labeled for 15 min with 80 μCi of [3H]leucine (New England Nuclear;
RESULTS
In our analysis of the virus repertoire present in Gross passage A virus extracts, we took advantage of a previous observation that ecotropic and dual tropic (MCF) MuLV can be distinguished by in NaDodSO\textsubscript{4}/polyacrylamide gel electrophoresis of their primary \textit{en} gene product (pr-env protein; previously designated PrENV protein) (21). The replication of ecotropic and dual tropic MuLV in the same cell can be detected biochemically by examination of MuLV \textit{en} gene products labeled in a short pulse (see Fig. 1) in spite of the fact that the dual tropic virus component may be masked virologically (refs. 26 and 27; unpublished data). Mouse SC-1 cells infected with AKR ecotropic virus (69E5) and AKR dual tropic virus (69L1) were pulse-labeled for 15 min with \textsuperscript{3}H\textit{I}leucine, cytoplasmic extracts of these cells were subjected to immunoprecipitation with rabbit anti-R-MuLV p15(E) serum, and immunoprecipitates were analyzed by NaDodSO\textsubscript{4}/polyacrylamide gel electrophoresis (Fig. 1, lane 2). Cells infected with either AKR 69E5 or AKR MCF 69L1 viruses also were subjected to the same procedure and the resulting immunoprecipitates were electrophoresed to serve as marker proteins (Fig. 1, lanes 1 and 3, respectively). The ecotropic virus pr-env protein and dual tropic virus pr-env protein are easily distinguishable by migration when isolated from cells infected with either virus alone or from coinfected cells.

Isolation of Viruses from Gross Passage A Virus Extracts. Virus was recovered in citro from two consecutive Gross passage A extracts (p104 and p105) by infection of either mouse SC-1 cells or mink CCL64 cells. Infected cells were pulse-labeled for 15 min with \textsuperscript{3}H\textit{I}leucine and cytoplasmic extracts were immunoprecipitated with goat anti-R-MuLV gp70 serum or rabbit anti-R-MuLV p15(E) serum. When the resulting immunoprecipitates were analyzed by NaDodSO\textsubscript{4}/polyacrylamide gel electrophoresis, two pr-env proteins were identified in infected SC-1 cells (Fig. 2A, lanes 3 and 4). One of the pr-env proteins expressed in infected SC-1 cells comigrated with G-MuLV pr-env proteins (Fig. 2A, lanes 2, 3, and 4) and the other pr-env protein comigrated with MCF 69L1 virus pr-env protein (Fig. 2A, lanes 3, 4, and 5). A single pr-env protein was expressed in infected mink cells and comigrated with that of MCF 69L1 virus (Fig. 2A, lanes 5 and 6). Partial protease digest mapping was used to compare the three pr-env proteins encoded by the recovered viruses with the pr-env proteins encoded by AKR ecotropic virus 69E5, G-MuLV, AKR MCF 69L1 virus, and a xenotropic virus induced by IdUrd from C3H/Bi mouse embryo cells (Fig. 2B). We have shown that peptide maps generated with \textit{S. aureus} V8 protease distinguish the pr-env proteins of ecotropic, xenotropic, and dual tropic (MCF) MuLV (28). The pr-env protein expressed in SC-1 cells infected by Gross passage A virus extracts that comigrates with that of G-MuLV is indistinguishable from the pr-env of G-MuLV by this mapping procedure (Fig. 2B, lanes 1 and 2). It should be noted that the map of G-MuLV pr-env protein is distinguishable from that of endogenous AKR ecotropic MuLV 69E5 (Fig. 2B, lane 3). The fast-migrating pr-env protein expressed in SC-1 cells infected by Gross passage A virus extracts generates a map that is similar but not identical with that of MCF 69L1 virus (Fig. 2B, lanes 4 and 5). The pr-env protein encoded by virus recovered from Gross passage A virus extracts in mink cells is indistinguishable from that of C3H/Bi endogenous xenotropic MuLV (Fig. 2B, lanes 6 and 7).

Thus, it appears from this analysis that three distinct viral genomes are present in extracts of Gross passage A virus and are recoverable in mouse or mink cells in citro: (i) an ecotropic component that is indistinguishable from G-MuLV isolated in 1965 by J. Hartley but distinguishable from endogenous AKR ecotropic MuLV, (ii) endogenous xenotropic MuLV, and (iii) a virus component that resembles the leukemogenic, \textit{en} gene recombinant virus MCF 69L1 isolated from an AKR thymoma.

Virus Expression in Leukemias Induced by Injection of Gross Passage A Virus Extracts. Leukemias were induced in C3H/Bi mice by two routes of injection, the classical route of i.p. injection into suckling mice and by i.t. injection into weanling mice. Suspensions of primary thymoma cells were pulse-labeled for 2 hr in citro with \textsuperscript{3}H\textit{I}leucine and MuLV pr-env proteins were isolated from cytoplasmic extracts by immunoprecipitation with anti-p15(E) serum and NaDodSO\textsubscript{4}/polyacrylamide gel electrophoresis. A series of five leukemias in-

FIG. 1. NaDodSO\textsubscript{4}/polyacrylamide gel electrophoresis analysis of pr-env protein of AKR ecotropic and dual tropic viruses. Cytoplasmic extracts were prepared from virus-infected SC-1 cells radiolabeled with \textsuperscript{3}H\textit{I}leucine in a 15-min pulse. Extracts were reacted with rabbit anti-R-MuLV p15(E) serum and resulting precipitates were subjected to electrophoresis in 7.5% acrylamide slab gels. Lane 1, ecotropic MuLV-infected cells; lane 2, cells coinfected with ecotropic and dual tropic MuLV; lane 3, dual tropic MuLV-infected cells.
produced by i.p. injection all expressed two MuLV pr-env proteins—one comigrating with that of G-MuLV and one comigrating with that of MCF 69L1 virus. Analysis of a representative leukemia is presented in Fig. 3A (lane 2). In addition, infection of SC-1 cells with cell-free extracts of these leukemias resulted in recovery of viruses that expressed both pr-env species (Fig. 3A, lane 3). Peptide mapping was used to compare the pr-env species expressed in the primary leukemias or encoded by viruses transmissible to SC-1 cells with those of G-MuLV and MCF 69L1 virus (Fig. 3A, lanes 5–9). In all instances, the map of the slow-migrating pr-env species was identical to that of G-MuLV and the fast-migrating pr-env species was similar, but not identical, to that of MCF 69L1 virus.

Analysis of five primary leukemias induced by i.t. injection revealed the expression of only one pr-env species that comigrated with that of MCF 69L1 virus. Analysis of a representa-
Leukemogenicity of GPA-V1 and GPA-V2 Virus Components Recovered from Gross Passage A Virus Extracts. Leukemias were induced in C3H/Bi mice by either GPA-V1 or GPA-V2 viruses that have been propagated in mouse SC-1 cells in vitro. Cell suspensions of the primary thymomas were pulse-labeled as described above and MuLV env gene products were isolated by immunoprecipitation and NaDodSO4/polyacrylamide gel electrophoresis. Three leukemias induced by i.p. injection of the ecotropic virus component GPA-V1 into suckling mice were analyzed. A representative leukemia is shown in Fig. 4 (lanes 2 and 3). Two pr-env proteins—one comigrating with the pr-env protein of the input GPA-V1 virus and one comigrating with the pr-env protein of MCF 69L1 virus—were expressed in these thymomas. The pr-env protein of MCF 69L1 virus and the fast-migrating pr-env protein isolated from the thymoma appear as doublets in Fig. 4. Formation of doublets in NaDodSO4/polyacrylamide gel electrophoresis is a property of dualtropic virus pr-env proteins that we have observed variably in the analysis of AKR virus isolates replicating in vivo and in vitro (28, 29). The molecular basis of this phenomenon is unknown. Injection of GPA-V1 by the i.t. route resulted in no leukemias to date (357 days postinjection), whereas the average latent period of leukemia induction observed after i.p. injection of GPA-V1 was 183 days. In contrast, leukemias were induced in C3H/Bi mice after injection of the dualtropic-like virus component GPA-V2 by either the i.p. or i.t. route with an average latent period of 184 days. One leukemia induced by i.p. injection and five leukemias induced by i.t. injection were analyzed biochemically. Representatives of leukemias induced by both protocols are shown in Fig. 4 (lanes 5 and 6). In all instances, leukemias induced by GPA-V2 virus expressed only a single pr-env protein that comigrated with that of the injected GPA-V2 virus and MCF 69L1 virus.

Extracts of leukemias induced by GPA-V1 and GPA-V2 viruses were used to infect SC-1 cells and these cells were analyzed for expression of MuLV pr-env proteins. Viruses encoding both the fast- and slow-migrating pr-env proteins were recoverable from leukemias induced by GPA-V1 virus alone (data not shown). Only MuLV encoding the fast-migrating pr-env species was recovered from extracts of leukemias induced by the GPA-V2 virus (data not shown).

Our findings suggest that expression of the dualtropic virus component GPA-V2 of the Gross passage A virus complex is necessary and sufficient for the induction of leukemias in C3H/Bi mice. In contrast, induction of leukemias by the ecotropic virus component GPA-V1 appears to involve generation of a second virus with characteristics of recombinant dualtropic MuLV.

**DISCUSSION**

It has been possible to identify three distinct MuLV components in Gross passage A virus extracts by exploiting several characteristics of their env gene products. Two viruses designated GPA-V1 and GPA-V2 have been isolated in mouse SC-1 cells and identified by the molecular weight in NaDodSO4/polyacrylamide gel electrophoresis and peptide maps of their pr-env proteins. GPA-V1 appears indistinguishable from the Xc′, ecotropic virus (G-MuLV) that was isolated in 1965 by J. W. Hartley from Gross passage A virus extracts and which has been analyzed extensively (19, 30, 31). GPA-V2 is an Xc′, dualtropic virus that appears related to AKR dualtropic (MCF) MuLV by the characteristic molecular weight and peptide map of its pr-env protein. Although GPA-V2 was infectious in vivo as evidenced by leukemogenicity of culture supernatants of infected SC-1 cells, the virus was poorly infectious in vitro. Virus transmission was demonstrated most efficiently by infectious center plating and showed a marked preference in host range for mouse SC-1 cells as opposed to mink cells (unpublished data). Thus, virological properties of GPA-V2 differed considerably from those of AKR dualtropic (MCF) MuLV (9, 14) and of dualtropic MuLV isolated recently by Hamada et al. (32) from Gross passage A virus-induced leukemias in NFS mice. The third MuLV component in Gross passage A virus extracts designated Gross passage A virus 3 (GPA-V3) was isolated in mink cells and was identified as xenotropic on the basis of host range and peptide map of its pr-env protein. Virological and serological properties of GPA-V1, GPA-V2, and GPA-V3 MuLV will be detailed in a separate publication.

Primary thymomas induced in C3H/Bi mice following i.p. injections of suckling mice with Gross passage A virus extracts have been shown to express the pr-env protein of both GPA-V1 and GPA-V2. Expression of the xenotropic MuLV component GPA-V3 was not detected in these cells. However, a low level of expression of this virus would be difficult to identify because its pr-env protein comigrates with that of GPA-V2. The frequency of GPA-V3 infectious centers in leukemia cell suspensions is very low relative to GPA-V1 and GPA-V2 infectious centers (unpublished data). Thus, GPA-V3 found in cell-free extracts may represent low level expression of endogenous C3H/Bi xenotropic MuLV by transformed cells or thymic stromal cells and be present adventitiously in the extracts.

Previous studies have shown that leukemogenic MuLV isolates induced disease with equal efficiency regardless of whether the route of injection was i.p. in suckling mice or i.t. in young adult mice (9, 14). This was also true for leukemia induction by Gross passage A virus extracts. However, examination of primary thymomas induced after i.t. injection showed that only the pr-env protein of GPA-V2 was expressed. Moreover, only GPA-V2 was recovered in SC-1 cells in vitro from cell-free extracts of these leukemias. Thus, varying the route of injection of Gross passage A virus resulted in the separation
of the XC⁻, dualtropic MuLV component GPA-V2 from the XC⁺, ecotropic MuLV component GPA-V1.

Once purified in vitro, the isolated GPA-V1 and GPA-V2 MuLV components of the Gross passage A virus complex were tested to assess the role played by each virus in the induction of leukemia as a function of the route of injection. Both GPA-V1 and GPA-V2 were leukemogenic in C3Hf/Bi mice. GPA-V1 induced leukemia with lower incidence and longer latency than Gross passage A extracts as reported previously (19, 20), but only when injected by the i.p. route. i.t. injection of GPA-V1 did not result in leukemia development—a finding which suggests that the target cell for transformation by GPA-V1 is not present in thymus. Primary thymomas induced by i.p. injection of GPA-V1 expressed both the pr-env of GPA-V1 and the pr-env of a virus that appeared to be identical to GPA-V2. Because no GPA-V2 could be demonstrated in the stocks of GPA-V1 (G-MuLV) that were obtained from J. W. Hartley and used in these experiments, we conclude that GPA-V2 was induced in vivo as a consequence of infection by GPA-V1 and suggest that previously reported leukemogenicity of the ecotropic MuLV component GPA-V1 is due to its capacity to induce GPA-V2-like virus. Thus, it would appear that the leukemogenicity of certain ecotropic MuLV such as GPA-V1 and also including Friend, Moloney, and Rauscher MuLV (8, 12, 15, 33) and AKR SL viruses (6) may derive from their capacity to induce expression of endogenous viral information with transforming potential. This is a property not shared by other endogenous MuLV like AKR 69E5 which was nonleukemogenic in our studies and those of others (6, 9, 14). Consistent with this hypothesis are preliminary studies that suggest that the virus-inducing capacity of GPA-V1 may not be restricted to the T cell lineage. Expression of a pr-env species similar to that encoded by GPA-V2 appears to be induced in mouse SC-1 cells in vitro by infection with GPA-V1 but not by AKR ecotropic MuLV. Results of these studies will be reported elsewhere.

The results of our analysis suggest that expression of GPA-V2 in thymocytes is essential for transformation. All leukemias examined were positive for GPA-V2 gene products. GPA-V2 that was propagated free of GPA-V1 in SC-1 cells induced leukemia with an incidence and latent period comparable to that of Gross passage A virus extracts from which this MuLV component was derived. Leukemia induction was independent of the route of injection, and only GPA-V2 pr-env protein was expressed in resultant leukemias.

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