Characterization of Virus Produced by a Lymphoma Induced by Inoculation of AKR MCF-247 Virus

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We report the characterization of the virus produced by a lymphoid cell line derived from a lymphoma of an AKR mouse after injection of the polytropic AKR virus MCF-247. The virus displays polytropic host range properties and is indistinguishable from MCF-247 as judged by analysis of the large RNase T₁-resistant oligonucleotides of the RNA genome. Restriction enzyme analysis of cellular DNA revealed the presence of sequences homologous to MCF-247 genomic RNA. The *Eco*RI cleavage fragments were characteristic of MCF-247 DNA provirus cleavage products.

Mice of the AKR strain developed thymus lymphoma at a high incidence between 6 months and 1 year of age (17). The disease is linked to the expression of endogenous retroviruses. The retroviruses which can be derived from tissues of AKR mice constitute a polymorphic group of agents. Viruses that have a host range restricted to mouse cells (ecotropic) and are positive in an XC fusion test can be isolated from AKR mice any time after 2 weeks of age (18). Viruses capable of replication on cells derived from animals other than mice (xenotropic) can be detected in older mice (8, 9). A number of other viruses can be isolated from the lymphoid tissues of preleukemic and leukemic mice. These include viruses that are negative in the XC fusion assay and that are ecotropic (6, 11, 12, 16) as well as viruses that have a broad host range, including both mink cells and mouse cells, and that are also negative in the XC fusion test (polytropic or MCF viruses) (4, 8).

These age-related changes in retrovirus expression may represent early events in the disease process. To test this hypothesis, viruses derived from the tissues of preleukemic and leukemic AKR mice have been injected into newborn animals to determine whether they accelerated the onset of disease. Results of such tests indicate that whereas the ecotropic XC⁺ virus that can be isolated from young animals does not accelerate the onset of disease when injected into newborn animals, several other viruses do (6, 12). These include some of the cloned polytropic isolates such as MCF-247 and

some of the viruses produced by cell lines derived from spontaneously occurring thymic lymphomas (SL viruses) (6, 11). For MCF-247 virus, the average age of incidence of thymic leukemia is decreased from 269 days to about 135 days (6) (see below).

Although injection of some strains of virus accelerates the onset of disease in AKR mice, a question remains as to whether the injected virus replicates in the tumor cells. To test this possibility, newborn AKR mice were injected with the polytropic MCF-247 strain of virus. A cell line was established from one of the thymic tumors which developed 5 months after injection, and the virus produced by these cells was analyzed.

Here we report that the virus produced by the tumor cells retained its polytropic host range and was indistinguishable from the strain used for inoculation as judged by a high-resolution analysis of the RNase T₁-resistant oligonucleotides of the RNA genome. Restriction enzyme analysis of virus-related sequences in the DNA of this lymphoma cell line indicates the presence of additional DNA cleavage fragments indistinguishable from MCF-247 DNA cleavage products.

MATERIALS AND METHODS

Virus. MCF-247 cloned virus (4) was provided by Janet Hartley. The virus used for inoculation was propagated in CCL-64 mink cells.

Cell lines. The cell lines used include a mink lung fibroblast line (CCL-64), the S⁺ L⁻ line (1) (kindly supplied by R. H. Bassin), the XC cell line (18), and a line of NIH-3T3 cells. A CCL-64 cell line infected with MCF-247 was used as a source of DNA for the restriction enzyme analysis of the MCF-247 provirus. The line was derived by plating single cells of an infected

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culture into 96-well Terasaki plates at a dilution of less than one cell per well. Wells receiving only a single cell were grown into large cultures, and one such line, AKR CL-4B, was used here. MCF-247 was also propagated in NIH-3T3 cells. Tumor cell lines derived from spontaneously occurring AKR thymic lymphomas include AKR SL1, SL2, SL3, SL5, SL6, SL7, and SL8 (10).

Mice. A strain of AKR/J mice was maintained by continuous, single brother and sister mating.

Establishment and growth of lymphoma cell line. The donor mouse developed thymic lymphoma 155 days after neonatal interperitoneal inoculation of 5×10^2 focus-forming units (FFU) of MCF-247 viruses per ml. Cells (5×10^6) from the thymus were placed in a 35-mm petri dish with Dulbecco-modified Eagle medium supplemented with 10% fetal bovine serum, 10 μ g of asparagine per ml, and 10^{-3} M glutathione. The nonadherent cells were transferred to new dishes twice a week. They became independent of glutathione after 5 weeks in culture. The cells were maintained in a suspension culture in Dulbecco-modified Eagle medium-10% total fetal calf serum without added glutathione for 2 months and then were changed to RPMI 1640 with 10% fetal bovine serum. Studies with the cell line were done after it had been in continuous culture for 6 months.

Virus assays. The activity of RNA-dependent DNA polymerase in tissue culture supernatant was determined as described elsewhere (19). The number of infectious viruses was determined by assessment of numbers of foci formed in S⁺L⁻ (D56) cells as described elsewhere (1). The XC cell fusion assay was performed by the method of Rowe and Pincus (18). The XC assays were performed on NIH-3T3 cells.

Isolation of virus RNA. Virus particles were isolated from tissue culture supernatants by centrifugation as described previously (13, 14). The viruses were disrupted by treatment with proteinase K (25 mg/ml in sodium dodecyl sulfate), and the 70S RNA was isolated by velocity sedimentation in sucrose gradient followed by chromatography on oligodeoxythymidylic acid-cellulose (13, 14).

Oligonucleotide mapping. The analysis of RNase T₁-resistant oligonucleotides of the viral RNA was carried out as described elsewhere (13, 14). Briefly, the 70S RNA was digested to completion by RNase T₁, and the RNase-resistant oligonucleotides were radioactively labeled by [γ -³²P]ATP and polynucleotide kinase and separated by two-dimensional gel electrophoresis. The oligonucleotide pattern (termed an RNase T₁ fingerprint) was visualized by autoradiography.

Synthesis of complementary DNA probe. Viral RNA purified from MCF-247 virions grown on NIH-3T3 cells was used for complementary DNA synthesis in a 100- μ l reaction that contained 10 mM Tris-hydrochloride (pH 8.3); 50 mM dithiothreitol; 0.01% Nonidet P-40; 1 mM each of dATP, dGTP, and dTTP; 6 mM MgCl₂; 215 mg of salmon sperm DNA primer per ml (7); [α -³²P]dCTP (Amersham-Searle, $\approx$ 600 Ci/mol), 90 U of avian myeloblastosis virus reverse transcriptase, and 4 to 5 μ g of viral RNA. Incubation was at 37°C for 2 h.

Restriction enzyme analysis of cellular DNA.

Cells were resuspended in 1 $\times$ SSC (0.15 M NaCl-0.015 M sodium citrate), sodium dodecyl sulfate was added to a final concentration of 1.0%, and tissues were digested with Proteinase K (10 μ g/ml) for 1 h at 37°C. Phenol saturated with 1 $\times$ SSC was used to extract the tissues twice, followed by four extractions with chloroform-isoamyl alcohol (19:1). Two volumes of cold ethanol were added to the aqueous phase, and the DNA was wound out. The DNA was resuspended in 1 $\times$ SSC and digested with RNase A (Worthington) at 40 μ g/ml for 30 min at 37°C. The DNA was then redigested with proteinase K as described above and re-extracted with phenol and chloroform-isoamyl alcohol. Two volumes of cold ethanol were added to the aqueous phase, and the DNA was wound out, lyophilized, and resuspended in 0.01 $\times$ SSC to an approximate concentration of 1 to 2 mg/ml.

Purified DNAs were digested with restriction endonuclease *Eco*RI (20 U/10 μ g of DNA, New England Biolabs). Digestions were performed at 37°C for 2 h in the cocktail suggested by the supplier. The DNA was subjected to electrophoresis on 0.8% agarose gels in a horizontal plate (14.5 cm by 21 cm by 6 mm) containing ethidium bromide at 3 μ g/ml. Electrophoresis was at 10 mA for 3 h followed by 25 mA for 16 h with constant current with a buffer of 0.04 M Tris-hydrochloride (pH 8.0), 0.018 M NaCl, 0.01 M sodium acetate, and 0.002 M Na₂ EDTA.

The DNAs were transferred from the agarose gels to nitrocellulose filters (Schleicher & Schuell, BA85) by a modification of the Southern procedure (20). The gels containing the DNAs were soaked in a denaturing solution of 1.5 M NaCl-0.5 M NaOH for 20 min and neutralized for 30 min with 3 M NaCl and 0 to 5 M Tris-hydrochloride (pH 7.0). After a 5-min soaking in 6 $\times$ SSC, the gels were placed on the nitrocellulose filters, and DNA transfer was allowed to proceed overnight at room temperature. The filters with the DNA were baked at 80°C for 4 h and stored until used.

³²P-labeled complementary DNA probes were added to DNA filters that had been preincubated in a solution containing 3 $\times$ SSC, 50% formamide, 0.5% sodium dodecyl sulfate, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 mM Tris-hydrochloride (pH 7.5), 1 mM Na₂ EDTA, 20 μ g of bovine rRNA per ml, and 20 mg of yeast RNA per ml. Approximately 5×10^5 cpm of probe having a specific activity of 10^7 cpm/ μ g were added in 0.5 ml of hybridization buffer. Hybridizations were carried out for 4 days at 37°C. Filters were washed for 16 h at 65°C in 2 $\times$ SSC-0.5% sodium dodecyl sulfate and then in 2 $\times$ SSC for 5 min at room temperature. Dried filters were autoradiographed at -70°C with Kodak XR film and an intensifying screen.

RESULTS

We wished to determine whether a virus that accelerated the onset of thymic leukemia in AKR mice replicated in the tumor cells. The MCF-247 virus derived from the thymus of a 7-month-old AKR mouse was used for this study. We selected this virus since it had been previously shown to accelerate lymphoma in AKR

mice (11). Moreover, the virus had been cloned and displayed a stable phenotype; it is polytropic and negative in the XC fusion test (4). To reduce the possibility that the virus used for the inoculation contained other murine viruses derived from the host cell, the MCF-247 was propagated on mink cells.

Our initial experiments confirmed the observation that MCF-247 accelerated the onset of leukemia when inoculated into newborn animals. Table 1 shows that the median age of onset of lymphoma in the inoculated animals was 135 days. AKR mice that had not been inoculated did not develop lymphoma before 155 days of age. The median age of onset for the spontaneous disease was 269 days. The gross pathology of the induced and spontaneous lymphomas were similar and included a greatly enlarged thymus in all mice, with enlargement of the spleen, the liver, and the lymph nodes in most of the animals.

Characterization of a cell line established from the tumor of a mouse injected with MCF-247. A cell line was established from a male animal injected with the MCF-247 virus that developed lymphoma at 155 days postinoculation. We designate this cell line AKR IL MCF-247-1, where IL is an abbreviation for induced lymphoma. The cells grow in suspension culture with a doubling time of 24 h. They are pleomorphic, some are round, and others have obvious pseudopods. The cells have the morphology of lymphoblasts as determined by Wright staining of cytocentrifuge preparations. They have basophilic cytoplasm without granules, and there is a prominent golgi zone. The nuclei are indented and have a fine homogeneous chromosome pattern and one to three nucleoli (Fig. 1). All of the lymphoid organs of the inoculated animals contained blast cells that effaced the normal architecture. These cells in culture produced low levels of virus when their supernatants were tested on S^+L^- cells (10 FFU/ml). However, supernatants tested on two occasions for RNA-dependent DNA polymerase were strongly positive (10^6 to 2×10^6 cpm/ml). All supernatants were from cultures that were in a logarithmic growth phase 3 days after transfer of new growth medium. When 10^6 cells were inoculated intraperitoneally into 2-month-old AKR mice, they produced generalized lymphoma within 14 days.

Host range of virus produced by AKR IL MCF-247-1 cells. The cell line produced virus that was detected by the activity of an RNA-dependent DNA polymerase in the supernatant and by focus formation on S^+L^- cells (10 FFU/ml). The cell supernatants were negative in the XC fusion assay.

TABLE 1. *Leukemia-accelerating properties of MCF-247 virus in AKR Mice^a*

Virus inoculated	No. of lymphomas before 155 days of age/ no. of mice	Median latent period (range)
MCF 247	17/26	135 (90-155)
None (alternate litter controls)	0/19	

^a The virus preparation was from CCL 64 (mink lung cell) supernatant fluid obtained from cells in growth phase. The supernatant contained 5×10^2 FFU of virus per ml.

Since the virus used for inoculation will replicate in both mouse cells and mink cells, we wished to determine whether the virus produced by the lymphoma cells also showed a dual host range. Cells from the thymus used to establish the lymphoma cell line were cocultivated with mouse and with mink fibroblasts, and the cell supernatants were used to reinfect both mouse and mink cells. As shown in Table 2, the lymphoma cells produced viruses that will replicate on mouse as well as on mink fibroblasts.

To determine whether the virus was polytropic, supernatant from the mouse cells that had been cocultivated with the lymphoma cells (for four subcultures) was used to inoculate mink cells. The mink cells infected with this virus produced virus that grows on mouse cells as judged by plaque formation on S^+L^- cells (37 FFU/ml). Supernatants obtained from mink cells cocultivated with the MCF-247-induced lymphoma cells also produced viruses after four subcultures that replicated on mouse cells as judged by plaque formation on S^+L^- cells (137 FFU/ml).

The viruses produced by either the mink or mouse cells cocultivated with these malignant thymocytes were negative in the XC fusion test. Virus in the supernatants from the AKR IL MCF-247-1 cell line also replicated on both mink and mouse fibroblasts and were negative in the XC fusion test. These observations are similar to those of Vogt (22), who demonstrated that thymic cells derived from an NIH mouse infected with a Moloney murine leukemia virus-related MCF virus produced an MCF-like virus, and those of Fischinger et al. (3), who demonstrated that polytropic viruses are found in lymphomas induced by HIX virus.

These experiments suggested that the virus produced by the AKR IL MCF-247-1 cell line was MCF-247 itself. However, this test does not rule out the possibility that the cell line produces a mixture of XC-negative ecotropic and xenotropic virus. Such mixtures can be maintained

FIG. 1. Wright stain of a cytocentrifuge preparation of AKR MCF-247-1 cells. $\times 400$.

TABLE 2. Virus studies of lymphoma cells from thymus used to establish AKR IL MCF-247-1 cell line^a

Indicator cell line	Reverse transcriptase (cpm/ml)		Host range
	Control	Test	
NIH 3T3 (mouse embryo)	578	141,710	Polytropic
CCL 64 (mink lung)	657	144,119	Polytropic

^a Thymus cell suspensions containing 10^6 cells were cocultured with indicator cells for 1 month (four subcultures). Supernatants were tested for reverse transcriptase activity and host range. Control is the reverse transcriptase assay value for uninfected cells. Host range was scored by the ability of the virus to grow on either mink (CCL64) or mouse (NIH 3T3) cell lines.

during growth on mink and mouse cells by phenotypic mixing of virion proteins (8, 22). However, we note that most of the tumor cell lines derived from thymic tumors that arise spontaneously in older AKR mice produce primarily ecotropic virus (12).

Fingerprint analysis of the AKR IL MCF-247-1 virus. Analysis of the large RNase T_1 oligonucleotides of genomic RNA provides a precise method for identification and discrimination of closely related viral strains. Therefore, we also applied this method to determine the relationship of the virus produced by the lymphoma line to the MCF-247 virus.

For this study, nonradioactive 70S RNA was isolated from MCF-247 virions produced by mink cells and from the viruses produced by AKR IL MCF-247-1. The RNA was digested with RNase T_1 , and the oligonucleotides were labeled with ^{32}P at the 5' termini and separated by two-dimensional gel electrophoresis. Figure 2a shows the oligonucleotide pattern for 70S RNA from MCF-247 grown on mink cells. The pattern is similar to the fingerprint obtained by Rommelaere et al. (15), except that the gel electrophoresis system used in the present study allows the detection of a larger number of oligonucleotides. The spot marked with an arrow represents an incomplete digestion product. As we have shown previously (14), the intensity of this particular spot depends upon the RNA concentration, and the oligonucleotide is not detected when the RNA sample is denatured before RNase digestion. In these experiments pictured in Fig. 2, this oligonucleotide provides a convenient marker for the presence of this RNA sample in the mixing test of Fig. 2c (see below).

Figure 2b shows the RNase T_1 fingerprint of 70S RNA of virus produced by the AKR IL MCF 247-1 cell line. The patterns in Fig. 2a and b are similar. Only the incomplete digestion product discussed above is clearly missing from the pattern of Fig. 2b. This is probably due to the low concentration of RNA used in the digestion reaction from this sample. To determine whether all of the oligonucleotides of the two

FIG. 2. RNase T_1 fingerprints of (a) 70S RNA isolated from MCF-247 virus grown on CCL-64 cells, (b) 70S RNA isolated from virus produced by the AKR IL MCF-247-1 cell line; (c) the RNase T_1 fingerprint of a mixture of equal amounts of the samples analyzed in a and b. The arrows in a and c point to the oligonucleotide which represents an incomplete digestion product.

fingerprints had the same electrophoretic mobility, portions of the two samples that contained equal amounts of radioactivity were mixed, and the pattern was analyzed by two-dimensional electrophoresis. The result is shown in Fig. 2c. This analysis shows that all the oligonucleotides of the RNA of the lymphoma line virus exactly comigrate with oligonucleotides of MCF-247 virus. With the one exception, discussed above, no oligonucleotide unique to MCF-247 could be detected. Comigrating oligonucleotides in this high resolution electrophoretic system will have exactly the same base composition, but not necessarily the same nucleotide sequence. However, the exact identity of the two patterns strongly implied that the comigrating oligonucleotides represent identical nucleotide sequences. Approximately 80 oligonucleotides representing 10 to 15% of the genome are resolved by this electrophoresis system. Therefore, we conclude that the major component in the cell supernatant of the lymphoma line is genetically identical or very closely related to the virus that was used for inoculation. The fingerprint pattern of Fig. 2b indicates that all the well-resolved, unique oligonucleotides are labeled at approximately the same intensity, and therefore must be present in approximately equimolar amounts (14). No other major RNA species was detected in this analysis. However, the method may not detect viral species that comprise less than 10% of the total population.

Detection of MCF-247 proviral DNA in the AKR IL MCF-247-1 cell line. To determine whether proviral DNA of MCF-247 could be detected in the AKR IL MCF-247-1 cell line, DNA was prepared from the cells and subjected to digestion by the restriction enzyme *EcoRI*. The restriction fragments were separated according to size by electrophoresis of the DNA on agarose gels. DNA fragments that contained sequences homologous to MCF-247 genomic RNA were identified by transfer of the DNA from the agarose gels to nitrocellulose filters and subsequent hybridization of the filters with radioactive cDNA made to MCF-247 genomic RNA. The pattern of restriction fragments that contained sequences homologous to MCF-247 was visualized by autoradiography.

EcoRI produces two DNA fragments (6.6 and 1.4 kilobases) that are internal to the provirus of MCF-247 (Fig. 3; Buchhagen, unpublished data). These fragments are not evident in the *EcoRI* restriction digest of the provirus DNA of the Akv virus (Steffen and Weinberg, personal communication; Lowy, personal communication; Buchhagen, unpublished data), nor is the 1.4-kilobase fragment evident as a major species in the *EcoRI* digest of the DNA of uninfected

young AKR mice (2, 21). Therefore, these fragments provide a convenient marker for infection of AKR tissues by MCF-247.

The experiments pictured in Fig. 4 demonstrated that both the 6.6- and 1.4-kilobase *EcoRI* DNA fragments are present in the DNA extracted from the AKR IL MCF-247-1 cell line (Fig. 4, lane 2). These DNA fragments have the same electrophoretic mobility as those produced by *EcoRI* cleavage of DNA purified from a mink cell line infected with MCF-247 (Fig. 4, lane 1).

FIG. 3. *EcoRI* restriction map of MCF-247 proviral DNA. The position of *EcoRI* sites in proviral DNA of MCF-247 virus integrated into the cellular DNA of a mink cell line is depicted (Buchhagen, unpublished data).

FIG. 4. *EcoRI* restriction analysis of AKR IL MCF-247-1 cellular DNA. Cellular DNA was prepared and subjected to restriction enzyme analysis as described in the text. Hybridization reactions included ^{32}P -labeled complementary DNA synthesized from MCF-247 70S RNA. The DNAs used were extracted from: AKR CL-4B, a cloned line of CCL-64 cells infected with MCF-247 (lane 1); AKR IL MCF-247-1 (lane 2); AKR SL7H (lane 3); AKR thymus from a 4-week-old mouse (lane 4); uninfected CCL-64 cells (lane 5); uninfected NIH-3T3 cells (lane 6). The molecular weight markers indicated at the left of each panel represent co-electrophoresis of *HindIII*-digested DNA (New England Biolabs) and from the top of the gel down are 23.0, 9.8, 6.6, 2.5 and 2.2 kilobases. The arrows indicate the positions of the AKR-247-specific *EcoRI* fragments and are 6.6 and 1.4 kilobases.

Such cleavage products are not evident in DNA extracted from the thymus of a 4-week-old AKR mouse (Fig. 4, lane 4), nor are they evident in other nonleukemic tissues of the AKR mice of any age that we have examined (data not shown). Moreover, DNA fragments of this length are not evident in the *Eco*RI digests of DNA purified from cell lines derived from spontaneous lymphoid tumors of the AKR mouse. Specifically, the 1.4-kilobase fragment is absent from *Eco*RI digest of DNA of the tumor cell lines AKR: SL1, 2, 3, 7, and 8 (for example, see Fig. 4, lane 3) as well as DNA of an established line of uninfected mink cells and uninfected NIH-3T3 cells (Fig. 4, lanes 5 and 6). From this data, we conclude that the AKR IL MCF-247-1 cell line contains more MCF-247 proviral sequences than are present in normal AKR tissues or that are present in tumor cell lines established from lymphomas that arise spontaneously in older animals.

DISCUSSION

The experiments presented here show that the virus produced by a tumor cell line derived from a tumor that arose from an animal injected with the polytropic virus MCF-247 produced a virus that was indistinguishable from the inoculated virus by both biological and biochemical tests. DNA of the tumor cells contained proviral sequences characteristic of the provirus of MCF-247 that were not evident in the DNA extracted from normal AKR tissues.

It is unlikely that the AKR IL MCF-247-1 virus represents an independent isolate of the MCF-247 virus. The fingerprint method used here distinguishes among closely related virus strains. In particular, the fingerprints of independent isolates of MCF viruses differ from one another in such an analysis. There are at least 10 differences in the unique oligonucleotides found in a comparison of the fingerprint of MCF-247 and MCF-13 (5; Pedersen and Haseltine, unpublished observations). Both viruses were derived from AKR mice. Fourteen differences in the unique oligonucleotides were found in the fingerprints of MCF-247 and MB34, an MCF virus derived from a strain of NIH mice that is partially congenic for the Akv-1 locus (5). Rommelaere et al. also detect differences among the fingerprints of MCF strains (15). Therefore, the identity of the fingerprints of the MCF-247 virus and the cell line AKR IL MCF-247-1 virus strongly suggests that the virus produced by the lymphoma line is identical to the inoculum.

The virus isolated from the induced lymphoma also differs from those produced by lymphoid cell lines derived from spontaneous tumors. Most of these isolates are ecotropic (10,

12, 15). Moreover, the fingerprints and biological properties of these viruses are substantially different from that of the MCF-247 (4, 15). The MCF-247-specific *Eco*RI restriction fragments are not found in cell lines established from spontaneously arising tumors in AKR mice, suggesting that the MCF-247 is not normally present in AKR tissues or tumors at levels detectable in these experiments.

From these experiments we conclude that the MCF-247 virus that accelerates the onset of thymic leukemia in AKR mice replicates in the tumor cells themselves. Therefore, it is likely that this virus plays a direct role in induction of the disease.

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