Proc. Natl. Acad. Sci. USA
Vol. 74, No. 7, pp. 2953–2957, July 1977
Genetics

Segregation of genetic information for a B-tropic leukemia virus with the structural locus for BALB:virus-1

(molecular hybridization/endogenous type-C RNA viruses/N- and B-tropic viruses/virus-induced leukemia)

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Communicated by David Baltimore, April 22, 1977

ABSTRACT **A B-tropic type-C RNA virus isolatable from lymphoreticular tumors of the inbred BALB/c mouse strain has previously been shown to be leukemogenic in its natural host. This virus is not chemically inducible from BALB/c embryo cells or from embryo lines containing segregating inducibility loci for two known endogenous type-C viruses of BALB/c cells. Molecular hybridization and type-specific immunologic assays demonstrate a high degree of genetic homology between the B-tropic leukemia virus and BALB:virus-1, an N-tropic endogenous virus of BALB/c cells. Genetic sequences specific for BALB:virus-1 are shown to segregate with the locus for BALB: virus-1 induction in genetic crosses between BALB/c and the noninducible NIH Swiss strain. Thus, if the information of the B-tropic virus is encoded in the genome of the animal, it must be closely linked to the structural locus for BALB:virus-1. The evidence is consistent with a mechanism by which a small genetic alteration in BALB:virus-1 leads to a virus, whose growth is unrestricted, and subsequently to the development of neoplasia.**

Type-C RNA viruses are known to cause naturally occurring tumors in several species (for review, see ref. 1). Evidence has accumulated that viruses with analogous morphologic and biochemical properties are genetically transmitted in a much larger number of species representing several vertebrate classes (1). As yet, only endogenous viruses of a few inbred mouse strains, selected for high incidence of leukemia (2, 3), have been demonstrated to be oncogenic for their hosts (4, 5).

It can be reasoned that the malignant potential of endogenous viruses of outbred species may be better understood by characterization of inbred mouse strains that more closely approximate the natural expression of malignancies of outbred populations. BALB/c has become a prototype of such strains with low to moderate tumor incidence. Its natural history of tumors and type-C virus expression are well documented (6–11). An as yet unresolved question emerging from these studies is the origin of a BALB/c (B)-tropic leukemogenic (10) virus that is often isolatable from late-appearing lymphoreticular malignancies of the BALB/c strain (10, 11). Extensive analysis of BALB/c cells has revealed the presence of several distinguishable endogenous viruses (8, 9), none of which possesses a host range like that of the B-tropic leukemia virus isolated *in vivo*. The present investigations were undertaken to study the origin of this leukemia virus of the BALB/c strain.

MATERIALS AND METHODS

Cells and Viruses. Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% (vol/vol) calf serum (Colorado Serum Co., Denver, CO) in 100 × 20 mm petri dishes (Falcon Plastics, Los Angeles, CA). Continuous mouse cell lines included BALB/3T3 (12), NIH/3T3 (13), and SC-1 (14). Secondary cultures of individual embryos of NIH Swiss (NIH), and BALB/c (BALB) parental genotypes as well as the (NIH × BALB)F_1 hybrid and NIH × (NIH × BALB)F_1 backcross generation have been described (8). Type-C viruses included inducible N-tropic and xenotropic endogenous viruses of the BALB/c strain, designated BALB:virus-1 and BALB: virus-2, respectively (8). A xenotropic endogenous virus of the NIH Swiss strain was also used (15, 16). These are prototypes of three distinguishable endogenous viruses present in many inbred mouse strains (1, 9). Independent isolates of B-tropic virus (3658, 3663, and 1290) from leukemic tissues of BALB/c mice were generously provided by R. Peters, Microbiological Assoc., Bethesda, MD.

Preparation of Cellular DNA. Cellular DNA was purified from cells by a modified Marmur procedure as described by Britten *et al.* (17). Nucleic acids were sheared to a mean size of 6–8 S at 50,000 rpm (−20°) in Virtis homogenizer (Virtis, Gardiner, NY).

Unique-sequence cellular DNA was prepared from NIH/3T3 cells grown for 24 hr in medium containing [^{3}H]thymidine (Schwartz/Mann, Orangeburg, NY) at 10 μCi/ml. ^{3}H-Labeled cellular DNA was extracted and sheared as described above, and highly reiterated DNA sequences annealing at a C_0t† of 50 mol·sec·liter^{-1} were removed by fractionation on hydroxyapatite as previously described (18).

Preparation of ^{3}H-Labeled cDNA. Viral [^{3}H]cDNA was prepared from sucrose gradient-purified virus as described (19, 20). The genetic complexity of viral cDNA was estimated by its ability to protect ^{32}P-labeled homologous 70S RNA from RNase digestion (21); at least 60% of the viral RNA genome was hybridized at a DNA/RNA molar ratio of 3. The specific activity of each viral DNA probe was 2×10^7 cpm/μg.

Hybridization. RNA·DNA hybridization was performed as described (20). For DNA·DNA hybridization, 0.5-ml reaction mixtures contained 4 mg of DNA, 12,000 cpm of [^{3}H]cDNA, 0.01 M Tris·HCl (pH 7.4), 0.6 M NaCl, and 1 mM EDTA. After heat dissociation, hybridization was performed at 62°. At varying times, 25-μl aliquots were quick-frozen and stored at −70°. Hybridization was assayed by the S1 nuclease method (22).

Radioimmunoassays for Viral Structural Proteins. The 12,000 molecular weight (p12) and 70,000 molecular weight (gp70) structural proteins of prototype endogenous mouse type-C viruses were isolated as previously described (9, 23). Each was labeled with ^{125}I at high specific activity (10 μCi/μg) by the chloramine T method of Greenwood *et al.* (24). The details of competition radioimmunoassay have been reported (9, 23).

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† C_0t = initial concentration of total DNA (mol of nucleotide/liter) × time (sec).

Table 1. Chemical induction of endogenous type-C viruses from embryo cell lines derived from genetic crosses between BALB/c and NIH mouse strains

Embryo culture	Virion-associated reverse transcriptase activity		
	SC-1	NIH/3T3	BALB/3T3
BALB/c	360	380	1.8
NIH	<0.2	<0.2	<0.2
(NIH × BALB)F_1	390	410	2.3
NIH × (NIH × BALB)F_1:			
B5	210	280	1.9
E8	180	140	0.6
E9	190	210	2.5
E1	310	350	6.0
E2	280	375	4.7
E4	110	150	1.0
A8	<0.2	<0.2	<0.2
B6	<0.2	<0.2	<0.2
B7	<0.2	<0.2	<0.2
E5	<0.2	<0.2	<0.2
E7	<0.2	<0.2	<0.2
E10	<0.2	<0.2	<0.2

Exponentially growing embryo cultures were exposed to IdUrd at 20 μg/ml for 18–20 hr, washed twice, trypsinized, and cocultivated with the indicated assay cells. At 2 and 6 weeks, tissue culture fluids were harvested, concentrated 100-fold, and assayed for poly(rA)·oligo(dT)-directed poly(dT) synthesis. Results are expressed as pmol of [^{3}H]dTMP incorporated per ml of tissue culture fluid and represent mean values of two separate determinations.

RESULTS

Attempts to Induce B-Tropic Virus Chemically from BALB/c Cells. The demonstration of the direct induction of a type-C virus from virus-negative clonal lines constitutes evidence of its genetic transmission (25, 26). The availability of NIH, BALB/c, (NIH × BALB)F_1, and NIH × (NIH × BALB)-F_1 embryo lines, previously characterized with regard to their inducibility of N-tropic and xenotropic viruses, made it possible to test these lines for inducibility of B-tropic virus with assay conditions known to detect virus induction from these cells.

As shown in Table 1, chemical induction of BALB/c or (NIH × BALB)F_1 embryo cells led to virus propagation at high titer in SC-1 and NIH/3T3 cells and to a much lesser extent in BALB/3T3 cells. When virus grown in BALB/3T3 cells was transmitted to new BALB/c and NIH cultures, it retained its N-tropic host range. Thus, if B-tropic virus were present in such stocks, it must represent only a minority of the total virus population. Induction of NIH cells failed to yield virus capable of growth in any of the assay cells tested.

Viruses induced from individual NIH × (NIH × BALB)F_1 backcross lines demonstrated growth patterns similar to those of viruses activated from (NIH × BALB)F_1 embryo cells. Viruses activated from lines previously shown to be inducible for the N-tropic virus BALB:virus-1 grew well in NIH/3T3 and SC-1 cells as compared to BALB/3T3. The host ranges of these viruses were N- rather than B-tropic. If an inducibility locus for B-tropic virus segregated independently from that of BALB:virus-1, induction of B-tropic virus might be expected from certain backcross lines not inducible for BALB:virus-1. However, as shown in Table 1, such backcross lines were consistently negative for B-tropic virus induction.

Biochemical and Immunological Relatedness of B-Tropic Leukemia Virus to BALB:Virus-1. To further investigate the origin of the B-tropic virus of the BALB/c strain, this virus was compared by molecular hybridization with several known endogenous viruses. As shown in Fig. 1, BALB:virus-1 cDNA was hybridized to extents of greater than 90% by RNAs of BALB:virus-1 and the B-tropic leukemia virus. In contrast, RNAs of two xenotropic viruses, BALB:virus-2 and the endogenous virus of NIH cells, hybridized no more than 60% of the same probe. Similarly, the B-tropic leukemia virus cDNA was hybridized more than 90% by its own RNA or RNA of BALB:virus-1 but no more than 60% by RNA of either xenotropic virus (data not shown). As controls, each xenotropic viral RNA was able to hybridize more than 90% of its respective cDNA (data not shown). These findings indicate a striking degree of genetic homology between the B-tropic leukemia virus and BALB:virus-1.

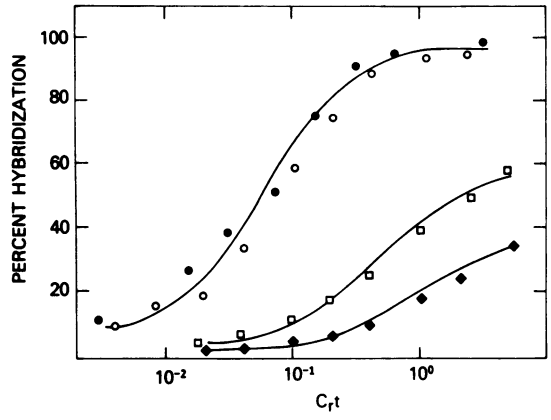

FIG. 1. Genetic homology of different endogenous mouse type-C viruses with BALB:virus-1. [^{3}H]cDNA prepared from BALB:virus-1 was hybridized by different viral RNAs: BALB:virus-1 (●); BALB:virus-2 (□); NIH murine leukemia virus (♦); and a B-tropic isolate of the BALB/c strain (3658) (○). C_rt = product of moles of RNA nucleotide per liter and seconds hybridized. No correction was made for Na^+ concentration.

Competition immunoassays for structural proteins have been very useful in studying relatedness among type-C viruses. This is particularly true for viral proteins such as p12 and gp70, which contain highly type-specific antigenic determinants (9, 23). In view of its marked genetic relatedness to BALB:virus-1, the B-tropic leukemia virus of the BALB/c strain was analyzed in immunoassays for BALB:virus-1 p12 and gp70. As shown in Fig. 2, BALB:virus-1 and a representative B-tropic virus isolate competed efficiently and to extents of more than 95% in immunoassays for BALB:virus-1 p12 and gp70. In contrast, none of the other viruses tested competed to more than an intermediate extent in either of these immunoassays. These results further demonstrate the high degree of relatedness of the B-tropic leukemia virus to BALB:virus-1.

Segregation of Genetic Sequences Specific for BALB:Virus-1 in Genetic Crosses between BALB/c and the Non-inducible NIH Strain. Previous molecular hybridization studies have demonstrated that a genetic locus for induction of an N-tropic virus of the AKR strain contains viral structural information of this virus (27). A similar approach was used to determine whether the locus for BALB:virus-1 induction contained its viral structural information and whether information for the highly related B-tropic virus segregated with this locus in the NIH × (NIH × BALB)F_1 backcross generation.

As shown in Fig. 3 and Table 2, the BALB:virus-1 cDNA probe demonstrated significantly greater sequence homology with BALB/c compared to NIH cellular DNA. BALB/c cellular DNA hybridized to more than 70% of the cDNA probe at a C_0t of 9×10^3. The change in the midpoint of the melting tem-

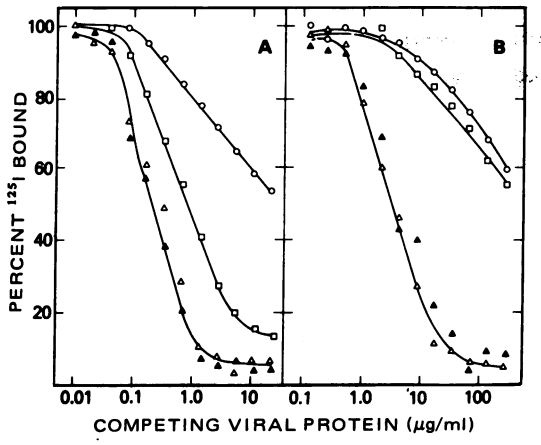

FIG. 2. Immunologic reactivities of different endogenous mouse type-C viruses in type-specific competition immunoassays for p12 (*A*) and gp70 (*B*) from BALB:virus-1. Detergent-disrupted viruses were assayed at 2-fold serial dilutions for their capacity to compete with ^{125}I-labeled antigen for limiting antibody. Results are expressed as the percentage of total ^{125}I-labeled antigen in the antigen–antibody precipitate standardized to 100% in the absence of competing antigen. The viruses tested included BALB:virus-1 (▲); BALB:virus-2 (□); NIH murine leukemia virus (○); and a B-tropic isolate of the BALB/c strain (3658) (△).

perature profile (Δt_m) was 1.1°, indicating a high degree of base pairing. In contrast, NIH cellular DNA was able to hybridize only 46% of this cDNA probe at the same C_0t value, and the Δt_m of the hybrid was much higher (5.5°). (NIH × BALB)F_1 cellular DNA also contained sequences specific for BALB: virus-1 cDNA, although reassociation kinetics indicated fewer viral copies than in the BALB/c parent. These findings indicated that BALB/c cellular DNA contained BALB:virus-1 sequences not present in NIH cellular DNA and, furthermore, that these same sequences were present in DNA of the F_1 hybrid.

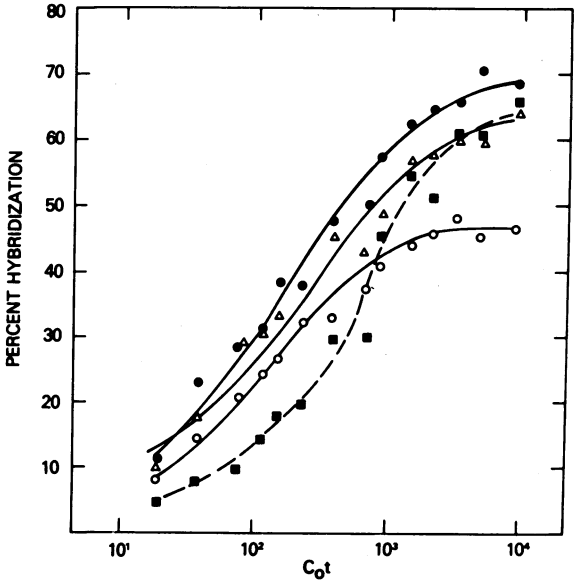

FIG. 3. Demonstration of BALB:virus-1 specific genetic sequences in BALB/c cellular DNAs by cell DNA–viral cDNA reassociation kinetics. BALB:virus-1 [^{3}H]cDNA was hybridized as described in *Materials and Methods* by cellular DNA prepared from: BALB/c (●); NIH (○); and (NIH × BALB)F_1 (△) embryo cells. Reassociation of unique mouse cellular DNA is also presented (■). No correction was made for Na^+ concentration. The detection limits of BALB: virus-1 specific nucleotide sequences in cellular DNA were approached when BALB/c cellular DNA was diluted 8-fold with NIH cellular DNA and annealed to BALB:virus-1 [^{3}H]cDNA. The mixture of cellular DNAs still hybridized the cDNA to a final extent of 7% more than did NIH cellular DNA.

Table 2: Segregation of structural information for N-tropic virus in individual NIH × (NIH × BALB)F_1 embryo cultures

Embryo culture	Chemically inducible virus* BALB: virus-1	BALB: virus-2	Maximum hybridization (%) of BALB: virus-1 cDNA†	Δt_m‡
BALB/c	+	+	71	1.1
NIH	−	−	48	5.4
(NIH × BALB)F_1	+	+	62	2.1
NIH × (NIH × BALB)F_1				
B5	+	−	66	1.4
E1	+	+	67	2.6
E2	+	+	70	2.3
E4	+	+	64	1.8
E8	+	−	69	1.7
E9	+	−	67	1.6
A1	−	−	41	8.3
A3	−	+	46	6.1
A8	−	−	42	7.0
B1	−	+	49	7.0
B6	−	+	49	6.8
B7	−	−	47	5.8
D8	−	−	45	4.6
E5	−	+	47	5.8
E7	−	−	45	4.9
E10	−	+	49	5.3

* Embryo lines were tested for chemically inducible ecotropic (BALB:virus-1) and xenotropic (BALB:virus-2) as described previously (8).

† Cellular DNA was extracted and hybridized to BALB:virus-1 cDNA probe as described in *Materials and Methods*. Results are expressed as the maximum levels of hybridization achieved at a final C_0t of 9×10^3 mol-sec-liter^{-1}.

‡ Thermal stability of hybrids formed between BALB:virus-1 cDNA and various cellular DNAs as measured by resistance to S1 nuclease. Δt_m is the difference in t_m between self-hybridizing labeled cell DNA molecules and viral cDNA-cell DNA hybrids, expressed in degrees centigrade.

Individual NIH × (NIH × BALB)F_1 backcross embryo lines were next analyzed for BALB:virus-1-specific genetic information. As summarized in Table 2, cellular DNAs of backcross embryo lines, known to be inducible for BALB:virus-1, each exhibited a significantly higher level of hybridization to BALB:virus-1 cDNA than that obtained with NIH cellular DNA. The levels of hybridization ranged from 64 to 70% (C_0t of 9×10^3). In contrast, DNAs of backcross embryo lines lacking BALB:virus-1 inducibility each hybridized no more than 49% of the cDNA probe under the same conditions, whether or not they contained the inducibility locus for BALB:virus-2, previously shown to segregate independently from that of BALB:virus-1 (8).

The t_m of hybrids formed between BALB:virus-1 cDNA and cellular DNAs of each of the backcross generation lines correlated with their known inducibility for BALB:virus-1 (Table 2). Thus, hybrids formed between the probe and cellular DNAs of lines that were inducible for BALB:virus-1 exhibited Δt_m values lower than those of lines negative for BALB:virus-1 inducibility. These findings establish that the inducibility locus for BALB:virus-1 contains structural information of this virus.

The results of analysis of cellular DNAs from the same parental and backcross generation embryo lines for genetic se-

quence homology with the B-tropic leukemia virus cDNA were identical to those obtained with BALB:virus-1 cDNA. Backcross lines inducible for BALB:virus-1 contained B-tropic viral genetic sequences not present in the noninducible parental line. If the locus for the B-tropic virus were not genetically linked to that of BALB:virus-1, 50% of the non-virus-inducible backcross embryo lines should have contained nucleotide sequences specific for the B-tropic virus. However, in no case was B-tropic virus-specific information detected in backcross lines that lacked inducible N-tropic virus. These findings indicate that genetic information of the B-tropic leukemia virus segregates with that of BALB:virus-1.

As a final test of the degree of relatedness of BALB:virus-1 and the B-tropic virus, several B-tropic virus isolates and N-tropic viruses, induced from individual backcross lines containing the structural locus for BALB:virus-1, were compared by molecular hybridization and immunoassay for their relatedness to BALB:virus-1. Table 3 demonstrates a high degree of genetic and immunologic homology among all of these viruses, providing further evidence of the close relationship between *in vivo* isolates of B-tropic virus and N-tropic viruses induced from individual embryo lines containing the structural locus for BALB:virus-1.

Table 3. Biochemical and immunological characterization of N-tropic virus isolates of individual NIH × (NIH × BALB)F_1 embryo cultures

Prototype endogenous virus	Maximum hybridization, %	Maximum competition, %†	
		p12	gp70
BALB:virus-1 (ecotropic)	95	97	96
BALB:virus-2 (xenotropic)	55	80	45
NIH-MuLV (xenotropic)	58	31	22
N-tropic virus induced from			
B5	93	95	98
E9	95	97	95
E1	94	98	95
E2	95	98	97
E4	90	96	97
B-tropic			
3658	96	97	99
3663	97	96	95
1290	94	95	96

* Viral RNAs were extracted and hybridized to BALB:virus-1 cDNA as described in *Materials and Methods*. Results are expressed as the maximum level of hybridization achieved at C_rt values of 10 mol·sec·liter^{-1} and represent mean values of three separate determinations.

† Homologous competition immunoassays for BALB:virus-1 p12 and gp70 were performed as described in *Materials and Methods*. Results are expressed as the percentage competition achieved at the highest viral protein concentration tested (0.02 μg/ml in p12 immunoassay and 1.0 μg/ml in gp70 immunoassay) and represent mean values from three separate determinations.

DISCUSSION

DNA reassociation techniques utilizing double-stranded cDNA probes obtained by reverse transcription of the mouse type-C viral genome led to the demonstration of multiple viral copies within mouse cellular DNA (28). Genetic studies have revealed that various inbred mouse strains contain different numbers of loci for induction of the same virus (29–31). Furthermore, within the BALB/c strain, loci for induction of biologically distinguishable viruses have been identified and shown to segregate independently (8). The application of methods using single-stranded viral cDNAs to the analysis of viral information present in different mouse strains made it possible to detect genetic sequences for a particular endogenous virus within cellular DNAs of some strains (32). In AKR, an inducibility locus for N-tropic virus, previously mapped within linkage group I of chromosome 7 (33), was found to contain sequences specific for this virus (27). These findings provided direct evidence that this inducibility locus contained viral structural information.

In the present studies, analysis of individual NIH × (NIH × BALB)F_1 backcross generation embryo cellular DNAs for genetic sequence homology with BALB:virus-1 defined its relationship to previously identified BALB/c virus inducibility loci. Only those cell lines that contained the inducibility locus for BALB:virus-1 demonstrated genetic sequences specific for this virus. This was the case whether or not the same cells were inducible for the xenotropic virus BALB:virus-2. These findings directly establish that the inducibility locus for BALB:virus-1 contains its structural information and, furthermore, that this structural locus segregates independently of the inducibility locus for BALB:virus-2.

A B-tropic leukemia virus of the BALB/c strain (10) was shown to exhibit a high degree of nucleotide sequence homology with BALB:virus-1. Moreover, in radioimmunoassays for two type-specific viral proteins, p12 and gp70, the two viruses were found to be indistinguishable. Previous reports have indicated small differences in the melting temperature profiles of homologous, as compared to heterologous, DNA·RNA hybrids of N- and B-tropic virus (34). Other studies have shown that N- and B-tropic viruses share more than 85% of their large RNase T_1 resistant oligonucleotides (D. Faller and N. Hopkins, personal communication), indicating relatively small differences in their nucleotide sequences. Slight differences have also been reported in the major structural antigens of N- and B-tropic viruses as determined by tryptic peptide analysis (35). Nonetheless, any genetic differences that exist between these two viruses are shown here to be minor in comparison to the marked biochemical and immunological differences that exist between either virus and other endogenous viruses of mouse cells.

The above findings have implications concerning the origin of the B-tropic leukemia virus. If this virus resides within the BALB/c genome, the present evidence establishes that its structural information must segregate with that of BALB:virus-1. The possibility must also be considered that the B-tropic virus, like several disease-inducing type-C viruses of other species (for review, see ref. 1), is not genetically transmitted but instead is an infectious agent acquired from some other source. Evidence against this hypothesis includes the striking biochemical and immunologic similarities between the B-tropic virus and BALB:virus-1, findings that favor their close evolutionary relationship. In other studies, efforts to demonstrate horizontal transmission of B-tropic virus from virus-positive BALB/c mice to littermates have not been successful (G. Kelloff, personal commication). Although milk-borne transmission of the B-tropic virus has not been directly excluded, epidemiologic studies indicate a lack of detectable B-tropic virus until well beyond the normal period of breeding. Thus, milk-borne infection is unlikely as the major mode of transmission of this virus.

If, as seems more likely, the B-tropic virus is genetically transmitted, it may exist as a discrete endogenous virus or arise as a variant of BALB:virus-1. By this model, the inability to

detect its activation from BALB/c parental or genetic crosses involving this strain would necessitate it being stringently regulated. Its late appearance in the animal might then occur only because of release from restriction in a particular differentiated cell type or as a result of a rare environmentally induced genetic alteration of a cellular regulatory mechanism.

Alternatively, the B-tropic leukemia virus may arise spontaneously as a variant of BALB:virus-1. The long latent period before detection of B-tropic virus *in vivo* might then be explained by an event of very low probability leading to virus conversion. To date, there have not been reports of N-tropic virus conversion to B-tropism in tissue culture, although NB-tropic variants have been observed after forced passage of either N- or B-tropic virus in nonpermissive cells (refs. 36 and 37; S. A. Aaronson, unpublished data). There is considerable evidence that the Fv-1 locus plays a major role in the growth restriction of these two host range variants. Fv-1 acts as a dominant inhibitor of virus infection (38, 39) at a postpenetration (40, 41) but preintegration step (42). BALB/c cells are known to be B-tropic virus permissive at Fv-1 (for review, see ref. 43), favoring the unrestricted growth of this virus when it appears *in vivo*. Thus, by either model of genetic transmission, the results would favor the concept that mouse strains, in addition to those selected for high tumor incidence, contain endogenous viruses whose malignant potential is dependent upon the interaction of the virus with host genetic controls.

This work was supported in part by Contract NCI-3-73-3212 from the Virus Cancer Program of the National Cancer Institute. The work by K.C.R. constitutes partial satisfaction of the requirements for a doctoral degree from the George Washington University.

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