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I am submitting herewith a thesis written by Russell Earl Hand, Jr. entitled "Host Range Analysis of RNA Tumor Viruses Isolated from Transformed BALB 3T3 Cells." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

Raymond W. Tennant, Major Professor

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HOST RANGE ANALYSIS OF RNA TUMOR VIRUSES ISOLATED FROM TRANSFORMED BALB 3T3 CELLS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Russell Earl Hand, Jr.

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To my wife Susan and my daughter Jennifer

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ABSTRACT

Mouse cells contain RNA tumor virus information integrated as DNA in their genome and can be induced to produce virus by treatment with the thymidine analogs 5-iodo-2'-deoxyuridine (IdU) and 5-bromo-2'-deoxyuridine (BrdU), inhibitors of protein synthesis or irradiation. Cultures of BALB 3T3 (clone A31) cells were treated with 100 μg/ml of IdU for 24 hours and after ten subcultures, 10^6 cells were found to produce tumors when inoculated subcutaneously in weanling BALB/c nude (athymic) mice.

The viruses produced by the induced A31 cells, designated as BXQ, were found by fluorescent antibody (FA) assay to infect a broad range of cells including mouse, rabbit (CCL-60), mink (CCL-64), and human cells (A_{204}) but did not produce XC plaques in any mouse cells tested. After several passages through mink cells, and three limit dilution isolations, a xenotropic virus was isolated; however, the mink cells also contained a virus which would infect mouse, rabbit, and human cells. When this source of virus was filtered and passaged through Swiss 3T3 cells to exclude the xenotropic virus the same broad host range and negative plaque pattern persisted. The host range of this apparent amphotropic virus remains stable through several passages in SC-1, CCL-60, and CCL-64 cells. This virus may be the result of the activation of an endogenous amphotropic type virus, or of a recombination between simultaneously induced endogenous exotropic and xenotropic viruses. To date the isolated viruses have not produced tumors when inoculated into newborn BALB/c nude mice.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	MATERIALS AND METHODS	8
	Cell Cultures	8
	Viruses	9
	Immunofluorescence Assay	9
	XC Plaque Assay	10
	Experimental Animal Inoculation	10
	Origin of BXQ Cell Line	11
III.	RESULTS	12
	Analysis of Virus from BXQ Cells	12
	Passage of Virus from BXQ Cells in Mink Cells	12
	Xenotropic Virus Purification by Limit Dilution	14
	Isolation of Amphotropic Virus	16
	Characterization of Amphotropic MuLV	19
	Fate of Amphotropic Virus in Mouse S3T3 and Mink CCL-64 Cells	19
	Inoculation of Cells Infected with Virus Isolates into Mice	22
IV.	DISCUSSION	25
LIST OF REFERENCES		31
VITA		36

LIST OF TABLES

TABLE		PAGE
1.	Host Range of Mammalian RNA Tumor Viruses	6
2.	Host Range of Virus from BXQ Cells	13
3.	Host Range of BXQ Virus After Passage in Mink Cells	15
4.	Purification of Xenotropic Virus by Limit Dilution	17
5.	Host Range Assay of Xenotropic Virus	18
6.	Host Range Assay of Amphotropic Virus	21
7.	Amphotropic Virus Host Range Pattern After Passage in S3T3 and Mink Cells	23

CHAPTER I

INTRODUCTION

In the 1930's several inbred mouse strains were developed which showed a high frequency of spontaneous leukemia. Work with the C58 strain (Richter and MacDowell, 1929) and various AK strains (Furth et al., 1933) showed that leukemia in these mice develops between 6 and 18 months of age, with up to 85% of the mice developing the disease. At the same time several mouse strains were developed which had a relatively low (1-2%) incidence of leukemia. One of these strains, C3H, had a low leukemia incidence but a high incidence (90%) of mammary cancer, Strong, 1935). Evidence that a virus was involved in the high incidence leukemia strains came from experiments in which cell-free extracts from AK leukemia cells were inoculated into day-old suckling C3H mice (Gross, 1951). Subsequently, over 50% of the inoculated mice developed leukemia compared to 1% for controls. Results of irradiation experiments with low incidence strains of mice suggested that a latent endogenous RNA tumor virus may be present in otherwise normal tissue. Irradiated C3H (Gross et al., 1959) and C57Bl (Lieberman and Kaplan, 1959) mice developed lymphatic leukemia and thymic lymphomas. Cell-free preparations of these tumors could induce the same type of neoplasm in non-irradiated C3H or C57Bl mice inoculated at birth. In vivo experiments established that murine leukemia was not only a viral disease, but that the virus is transmitted vertically through the germ cells. Cross-breeding experiments using high incidence (100%) AKR males with low incidence (<1%) C3H females led to an approximately 50% incidence of leukemia in progeny mice (Gross and Dreyfuss, 1967).

When small numbers of AKR embryo cells were plated in tissue culture, cell lines were established which would spontaneously produce virus. However, two of the cloned sublines remained negative for virus expression but had the capacity to produce virus either spontaneously, with treatment by ultraviolet or X-irradiation or transformation by SV40 virus (Rowe et al., 1971). These in vitro studies indicated that all AKR cell lines carry the full viral genome in an unexpressed form, that is, that they are endogenous to mouse cells.

Studies with BALB/c mouse embryo cells presented the possibility that the genetic information for producing murine leukemia virus (MuLV) is present in a repressed form in every mouse cell. After several months in culture, certain BALB/c clones began to release mouse leukemia virus and produce tumors when inoculated into newborn or irradiated weanling BALB/c mice (Aaronson et al., 1969). One interesting characteristic of the spontaneously released endogenous virus was that it grew very poorly in BALB/c embryo cultures - the cells from which it emerged.

The horizontal spread of activated endogenous MuLV is regulated by host genes which also control exogenous viral infection. The chief host-range control is exerted by the Fv-1 locus (Pincus et al., 1971a,b) which determines whether cells are permissive to the replication of N-tropic or B-tropic viruses. Using the XC plaque assay they were able to classify embryonic cells from 12 mouse strains as N-type or B-type. Hartley et al. (1970) found that the differences in host response to infection with N- or B-tropic viruses were relative, not absolute. N-tropic viruses initiated infection more efficiently on N cells than on B cells, whereas B-tropic viruses showed a reciprocal pattern. Moloney MuLV, a laboratory

strain of virus, infected both cell types equally well and was designated NB-tropic. A cell line has been derived by clonal selection from a feral, wild, mouse embryo cell culture which lacks the Fv-1 function (Hartley and Rowe, 1975). This cell line, designated SC-1, is equally permissive for N-tropic and B-tropic MuLV and can support the replication of xenotropic MuLV to a limited extent.

Some endogenous murine viruses do not fit the N-B host range pattern. One unusual virus which is carried by NZB mice (Mellors and Huang, 1966; East et al., 1967) can rescue MSV from transformed hamster cells, infect normal hamster and rat cells, but not N- and B-type mouse cells (Levy and Pincus, 1970). Other such endogenous viruses termed "xenotropic" have been found in a variety of mouse cells (Levy, 1973; Todaro et al., 1973). This new major host range class of MuLVs has been described as a group of viruses which are unable to replicate in any mouse cells so far tested, but are able to replicate well in heterologous species such as rat, rabbit, mink, monkeys, and humans. These viruses can now be isolated from a large number of inbred mouse strains, including NIH Swiss, BALB/c, and NZB (Levy, 1973; Todaro et al., 1973).

Rigorous study of the events involved in MuLV induction and expression has been hampered because of the complexity of the in vivo systems, and the emergence of MuLVs in vitro is often a rare event in many cell lines. AKR cell lines which are virus negative but capable of spontaneous MuLV production can be induced to produce virus by treatment with the halogenated pyrimidines 5-iodo-2'-deoxyuridine (IdU) and 5-bromo-2'-deoxyuridine (BrdU) (Teich et al., 1973). By exposing AKR cells to 20 or 100 μg/ml of IdU or BrdU for 24 or 48 hours as many as 0.5% of the

cells can be induced to synthesize MuLV. This represented a 10^6-fold increase in production as compared to the spontaneous rate (Lowy et al., 1971). Aaronson et al. (1971) treated BALB/c embryo clones, particularly the BALB/3T3 clone A31, with IdU and BrdU and measured the induction of MuLV type-C virus by the sensitive reverse transcriptase enzyme assay. In most studies the viral enzyme activity followed a characteristic time course, with an early peak of virus production and then a progressive decline to a level below detection. Virus production was also measured by transferring fluids to NIH/3T3 and BALB/3T3 cells and applying the XC plaque assay. Plaque formation was at least 200 times more efficient on NIH/3T3 than on BALB/3T3, suggesting an N-tropic MuLV.

BALB/3T3 cells, when treated with BrdU, will also release a xenotropic virus in large excess over the N-tropic virus (Aaronson and Stephenson, 1973). Xenotropic virus is selectively induced if the cells are treated with protein synthesis inhibitors, in particular cycloheximide (Aaronson and Dunn, 1974). It is possible that the drugs specifically interfere with a labile protein whose function is to prevent virus synthesis, and may act either before or after transcription. Therefore, the BALB/c mouse cell contains at least two endogenous viruses which can be differentially induced by treatment with chemicals. The N-tropic virus (BALB:virus-1) is inhibited from spreading to other BALB/c cells because of the Fv-1 gene, while the xenotropic (BALB:virus-2) is prevented from propagating in BALB/c cells (or any other mouse cells) by an as yet undefined mechanism of restriction. Another characteristic of the two viruses is that only the BALB:virus-1 will trigger the XC plaque response when assayed on permissive cells (Aaronson and Stephenson, 1973; Levy, 1973).

Recently a third major host range class of endogenous RNA C-type virus isolated from wild Mus musculus has been described. These viruses, designated "amphotropic", replicate in mouse, rabbit, mink, human, guinea pig, and rat cells, but not in hamster, quail, or duck cells. Cultures of wild mouse cells contain a mouse-tropic virus which is positive in the XC plaque assay, in addition to the amphotropic virus which does not trigger an XC cell response. A xenotropic virus was never detected in any of the wild mouse cell lines tested. All of the amphotropic viruses tested showed N-tropism for mouse cells, contain MuLV group specific (P30) antigen, reverse transcriptase, and do not interfere with xenotropic or mouse tropic (ecotropic) viral infection (Hartley and Rowe, 1976; Rasheed et al., 1976; Bryant and Klement, 1976). The amphotropic virus could represent an ancestral virus from which mouse-tropic and xenotropic viruses were derived or it could be the result of the recombination between mouse ecotropic and xenotropic viruses. To date an amphotropic virus has not been isolated from a laboratory mouse cell line.

In summary, the three classes of endogenous viruses contain the same group specific (P30) antigen and reverse transcriptase but they can be differentiated on the basis of their host range infectivity patterns (Table 1). In the studies presented in this thesis BALB/3T3 clone A31 cells were treated with IdU, allowed to grow to confluency and subcultured to allow for maximum endogenous virus expression. When cells were inoculated into newborn BALB/c nude mice, solid tumors developed. To determine if the tumorigenicity of these cells was due to the virus(es) being produced, viral isolation procedures were initiated. This report describes the separation and isolation of a xenotropic and amphotropic

TABLE 1

Host Range of Mammalian RNA Tumor Viruses

Cells	Viruses				
	Mouse-Tropic				
	N-Tropic	B-Tropic	NB	Xenotropic	Amphotropic
Mouse					
$Fv\text{-}1^{n/n}$ (S3T3)	++++[a]	+	++++	-	++++
$Fv\text{-}1^{b/b}$ (BALB/c)	+	++++	++++	-	+
$Fv\text{-}1^{-/-}$ (SC-1)	++++	++++	++++	±	++++
$Fv\text{-}1^{n/b}$ (NIH Swiss x BALB/c)f_1	+	+	+	-	+
Rat (NRK)	++	++		++	++
Rabbit (CCL-60)	-	-	-	++++	++++
Mink (CCL-64)	-	-	-	++++	++++
Human (A_{204}, WI-38)	-	-	-	±	++++
Hamster	-	-	-	-	-
Quail, Duck	-	-	-	++	-
Baboon	-	-	-	++	

[a]Relative susceptibility 1+ = approximately 10% of cells infected at moi $\geq$ 1.

virus on the basis of host range properties and applied a methodology for separation of such viral mixtures using limit dilution purification and cell selective propagation.

CHAPTER II

MATERIALS AND METHODS

Cell Cultures

NIH Swiss (SME) (Microbiological Associates, Bethesda, Md.) and BALB/c (Cumberland View Farms, Clinton, Tn.) mouse embryo cultures were grown and maintained in 10% fetal bovine serum (FBS) (Microbiological Associates) in Eagles minimal essential medium (EMEM) (Grand Island Biological Company, Grand Island, N.Y.).

Continuous passage lines used for virus propagation and assays were S3T3 cells, an NIH Swiss culture developed in this laboratory; BALB/c 3T3 clone A31 (Dr. Stuart Aaronson), SC-1 cells, a clonal line of wild mouse embryo (Hartley and Rowe, 1975), a human rhabdomyosarcoma cell line A_{204}, and the rabbit corneal cell line SIRC (ATCC CCL-60). Two baboon cell lines; fetal skin and muscle (FSM) and whole baboon embryo (WBE) were obtained from Dr. R. L. Heberling (Southwest Foundation for Research and Education, San Antonio, Texas). Primary Japanese quail embryo (QE) cells were cultured in this laboratory. The cultures were maintained on McCoy's 5A media with 10% FBS. For virus assays the media was supplemented with 5% FBS and 50 μg/ml gentamicin. Mink lung cells (ATCC-CCL-64) and the mink lung F648 S^+L^- cell line both obtained from Dr. Paul Peebles (National Institutes of Health, Bethesda, Md.) were maintained and used in virus assays in RPMI-64 media supplemented with 5% FBS and 50 μg/ml gentamicin.

Viruses

The origin and passage history of the isolated viruses are described in the text. Strains of mouse-tropic MuLV used were Moloney (MuLV), N-tropic Gross strain, and B-tropic strain WN1802B obtained from Dr. Janet Hartley (National Institutes of Health).

Immunofluorescence Assay

For fluorescent antibody assay, cells were grown on 11 x 22 mm glass coverslips (Bellco Glass Co., Vineland, N. J.) in 60 mm plastic petri dishes (Falcon Plastics). After 12-18 hours the cells were treated with 25 μg/ml DEAE-dextran (Pharmacia Upsala) for one hour at 37°C, washed once, infected with virus for two hours and refed with complete media. After 48 hours the cells were washed once in Dulbecco's modified (Dulbecco and Vogt, 1954) phosphate buffered saline (PBS), fixed in cold acetone for 20 minutes, air dried, and stored at -20°C. Coverslips were then stained with fluorescein isothiocyanate-conjugated goat antiserum prepared against disrupted Moloney MuLV (Dr. Roger Wilsnack, Office of Program Resources and Logistics, Viral Oncology, National Cancer Institute), in diluted Rhodamine counterstain, at 37°C for one hour. To eliminate nonspecific staining, the antiserum was incubated at 37°C for one hour with a 10^7 cells/ml mixture of acetone fixed mouse, rabbit, and mink cells, and then centrifuged in a Sorvall HB4 rotor at 10,000 rpm (16,300 x g). Stained coverslips were washed in PBS for five minutes, distilled water for five minutes, air dried, and then mounted in a drop of water on a microscope slide.

Fluorescent cells were counted with a Zeiss microscope which had an Osram mercury vapor lamp, BG 12 exciter filter, and 500 nm barrier filter.

Positive fluorescent cells, based on 10 fields or 10^3 cells counted, were expressed as a percentage of the total cell population counted.

XC Plaque Assay

Viral infection was also measured by the UV-XC plaque assay (Rowe et al., 1970). Appropriate assay cells were plated in 35 mm Linbro dishes (Linbro Chemical Company, Hamden, Conn.) at 1 x 10^5 cells/well. After 18-24 hours the cells were treated with 25 μg/ml DEAE-dextran in McCoy's media for one hour, washed once, and inoculated with either 0.2 or 0.5 ml of the appropriate virus dilution. After four days all cells, except SC-1 cells, were exposed to 1800 ergs/sec/mm^2 of ultraviolet light (General Electric germicidal lamp). SC-1 cells were exposed to 1500 ergs/sec/mm^2. The cells were then overlayed with 4 x 10^5 rat XC cells/well in complete McCoy's media containing 2% FBS. After 24 hours the cells were washed once and refed with complete McCoy's media with 2% FBS. When the XC cells became confluent, usually within the next 24 hours, they were fixed and stained with a mixture of methanol, methylene blue, and carbol fuchsin. Plaques were scored under low microscopic magnification and only those plaques containing two or more large syncytial cells were counted as positive.

Experimental Animal Inoculation

Pregnant BALB/c mice, heterozygous for the athymic nude genotype, were obtained from Life Sciences, Inc., St. Petersburg, Florida. Newborn mice were inoculated with 10^6 BALB/c clone A31 cells per 0.1 milliliter containing the amphotropic isolate or 5 x 10^6 mink CCL-64 cells per 0.1 milliliter containing the amphotropic and xenotropic viruses. Newborn mice were also inoculated with the appropriate control cells.

Origin of BXQ Cell Line

Initial studies for the derivation of the BXQ cell from BALB/c clone A31 cells were performed by Fred Myer and John Quarles under the direction of Raymond W. Tennant.

Cultures of mouse BALB/3T3 clone A31 cells were held in medium containing 100 μg/ml of IdU for 24 hours; they were then washed and held for two weeks to allow the cells to overcome cytotoxicity and reach confluency. After the cells were subcultured four times, supernatant fluid from those cells was transferred to mouse (SME and BALB/c) and rabbit cells, held for 24 hours, and then assayed for viral proteins by the fluorescent antibody assay. Immunofluorescent positive cells were detected in all of the cell lines tested, indicating the replication of viruses with at least N- and xenotropism. The infected rabbit cell line was serially subcultured with the intention of making a pure xenotropic virus pool. Virus from daily medium collections was consistently positive in the fluorescent antibody assay on rabbit cells and did not form XC plaques when fluids from these cultures were used to inoculate SME and BALB/c cells.

Cells from passage ten of BXQ cells were inoculated (10^6 cells per mouse, subcutaneously) into young weanling BALB/c (athymic) nude mice as a test for tumorigenicity. After six months all of the mice receiving the BXQ cells had developed tumors at the site of inoculation, while those receiving untreated A31 cells did not. When the BXQ cells were tested for tumorigenicity at the 15th passage the time required for all of the inoculated mice to develop tumors was reduced to two months.

CHAPTER III

RESULTS

Analysis of Virus from BXQ Cells

Culture medium from the BXQ cells at passage 12 and passage 31 were examined by immunofluorescence and XC plaque assays, and virus which was infectious for a broad host range of cells was found, which would again indicate the presence of at least an N-tropic and xenotropic virus (Table 2). However, the virus did not induce the XC plaque response, and this is consistent with reports that some activated endogenous viruses of the N-tropic type did not produce plaques when assayed on SME and SC-1 cells (Hopkins and Jolicoeur, 1975; Rapp and Nowinski, 1976). In general, type C leukemia viruses do not transform fibroblast-like cells in culture or produce tumors in mice although they readily infect and replicate in them. On the other hand, type C sarcoma viruses transform cells in culture and produce tumors when inoculated into susceptible mice. However, because sarcomagenic viruses have not been isolated from IdU treated cells, studies were undertaken to isolate and characterize the endogenous viruses in the BXQ cell line, and to determine if they had any relation to the tumorigenicity of the cells.

Passage of Virus from BXQ Cells in Mink Cells

It was reasoned that the mouse-tropic (ecotropic) component of the virus mixture could be eliminated by infecting CCL-64 mink cells, since these cells are highly permissive for xenotropic virus, but are non-permissive for the ecotropic virus strains Gross type (N-tropic) and WN1802B (B-tropic) MuLV at multiplicities (viruses/cell) of infection (MOI)

TABLE 2

Host Range of Virus from BXQ Cells

Cells	Virus			
	Passage 12		Passage 31	
	Fluorescent Cells (%)[a]	XC pfu[b]/Well	Fluorescent Cells (%)	XC pfu/Well
Mouse (SME)	82	0	36	0
Mouse (BALB/c)	36	0	32.6	0
Mouse (SC-1)	96	0	88	0
Rabbit (CCL-60)	95	NT	73	0
Human (A_{204})	7	NT	3.5	NT[c]
Human (WI-38)	NT	NT	.37	NT

[a]Percent positive based on 10 fields or 10^3 cells counted.

[b]Cells inoculated with 0.5 ml of virus harvested after 18 hours.

[c]Not tested.

greater than one. Filtered supernatant fluids were inoculated onto subconfluent mink cell cultures. The cells were subcultured once, and then this culture media was filtered and used as a source of virus to infect more mink cells. The filtration procedure was used to eliminate both intact BXQ cells and also large virus aggregates from the medium. The infected mink cells were then subcultured (four passages over a period of four weeks) until all of the cells were expressing viral antigens by immunofluorescence assay.

To test the host range of this virus, a fresh harvest of culture fluid, collected 18-24 hours after refeeding, was inoculated onto SME, BALB/c, and SC-1 mouse cells, rabbit CCL-60, and human A_{204} cells. As shown in Table 3 the virus again infected a broad host range of cells but still did not form XC plaques. This infectivity pattern remained consistent through 19 subcultures. Because xenotropic virus grows well in mink cells, and no known endogenous ecotropic virus has been demonstrated to infect these cells, it was apparent that a possible amphotropic-type virus was present, possibly together with a xenotropic virus. To purify the virus(es), limit dilution inoculations were done to separate the major virus from a possible contaminating virus.

Xenotropic Virus Purification by Limit Dilution

Both CCL-64 mink cells and the transformed S^+L^- mink F648 cells are permissive for xenotropic viruses. Therefore, 10-fold dilutions of culture medium containing virus were added to duplicate plates of each cell type. The end point dilution was determined by the appearance of foci on the S^+L^- cells at the end of seven days. The normal mink cell cultures infected with the next highest dilution of virus beyond the focus end

TABLE 3

Host Range of BXQ Virus After Passage in Mink Cells

Cells	Virus			
	Passage 5		Passage 18	
	Fluorescent Cells (%)[a]	XC pfu[b]/Well	Fluorescent Cells (%)	XC pfu/Well
Mouse (SME)	27.5	0	49	0
Mouse (BALB/c)	7.5	0	4	0
Mouse (SC-1)	81.0	0	75	0
Rabbit (CCL-60)	61.3	0	90	0
Human (A_{204})	2.3	NT[c]	NT	NT

[a]Immunofluorescent.

[b]XC plaque forming units/well.

[c]Not tested.

point in S^+L^- cells was subcultured until all cells were producing virus. This virus was then assayed by immunofluorescence on mouse SME, BALB/c, SC-1, rabbit CCL-60, and human A_{204} cells to test the host range specificity. The virus initially infected only the rabbit CCL-60 and human A_{204} cells, but after two additional subcultures virus from the culture medium was able to infect the SME and SC-1 as well (data not shown). These results indicated that the xenotropic virus was in excess, but not enough to be separated from the putative amphotropic virus by 10-fold dilution steps. Therefore, in the next purification procedure the virus was diluted in 10-fold steps to 10^{-3}, where further dilutions were made in 2-fold increments. The cells containing the next highest dilution beyond the focus end point were subcultured several times before another limit dilution purification was carried out (Table 4). As shown in Table 5 the virus which was purified by this limit dilution had a host range consistent with xenotropic BALB:virus-2 isolated by others, in that it would not productively infect N-type or B-type mouse cells when assayed by immunofluorescence or induce an XC plaque response in any mouse cell culture tested.

Isolation of Amphotropic Virus

Based on the initial attempts at limit dilution purification, the putative amphotropic virus appeared to be present in approximately equal proportion to the xenotropic virus in the mink cells. Because xenotropic virus does not infect N-type mouse cells, the isolation of the amphotropic virus was carried out by inoculating S3T3 cells with a 1:10 dilution of filtered virus mixture from the mink cells. Two days following inoculation the culture medium containing virus was collected, filtered,

TABLE 4

Purification of Xenotropic Virus by Limit Dilution

Second Limit Dilution (Reciprocal Log_{10})	FFU[a]	Third Limit Dilution (Reciprocal Log_{10})	FFU
3.0	181		
3.3	47		
3.6	24		
3.9	16		
4.2	5	4.0	66
4.5	2	4.3	37
4.8	1	4.6	20
5.1	0	4.9	9
		5.2	0

[a]Focus forming units assayed on F648 (mink S^+L^-) cells.

TABLE 5

Host Range Assay of Xenotropic Virus

Cells	Fluorescent Cells (%)	XC pfu[a]/Well
Mouse (SME)	0	0
Mouse (BALB/c)	0	0
Mouse (SC-1)	24	0
Rabbit (CCL-60)	72	NT[b]
Human (A_{204})	>1	NT
Baboon	50	NT
Quail	54	NT

[a]Plaque forming units.

[b]Not tested.

diluted 1:10 and one milliliter was inoculated onto another plate of S3T3 cells. These cells were then subcultured until all of the cells became positive by immunofluorescence assay (three passages). To avoid the transfer of virus producing S3T3 cells, supernatant fluid from this culture was filtered and used to infect SC-1, CCL-60, and CCL-64 cells (Fig. 1). When these cells were subcultured five times the virus from each culture was harvested and the host range determined by infecting SME, BALB/c, SC-1, CCL-60, A_{204}, Baboon and quail embryo (QE) cells. As shown in Table 6 the virus from the three cell lines, after the isolation procedure, was capable of infecting a broad host range of cells as assayed by immunofluorescence. The XC plaque response when the virus was inoculated onto mouse cells was consistently negative. These patterns have remained stable on subsequent serial passage of the infected mouse SC-1, rabbit CCL-60, and mink CCL-64 cells. However, unlike the amphotropic virus isolated by Hartley and Rowe (1976) this virus, isolated from IdU treated A31 cells, was able to grow in quail embryo cells.

Characterization of Amphotropic MuLV

The amphotropic virus was identified as a member of the Retravirus family on the basis of reactivity with group-reactive antiserum in immunofluorescence tests, reverse transcriptase activity, and the ability to rescue the Kirsten sarcoma genome from KNRK cells to form an amphotropic pseudotype.

Fate of Amphotropic Virus in Mouse S3T3 and Mink CCL-64 Cells

As described previously, the amphotropic virus was isolated by two passages in the mouse S3T3 cells. These cells were serially subcultured

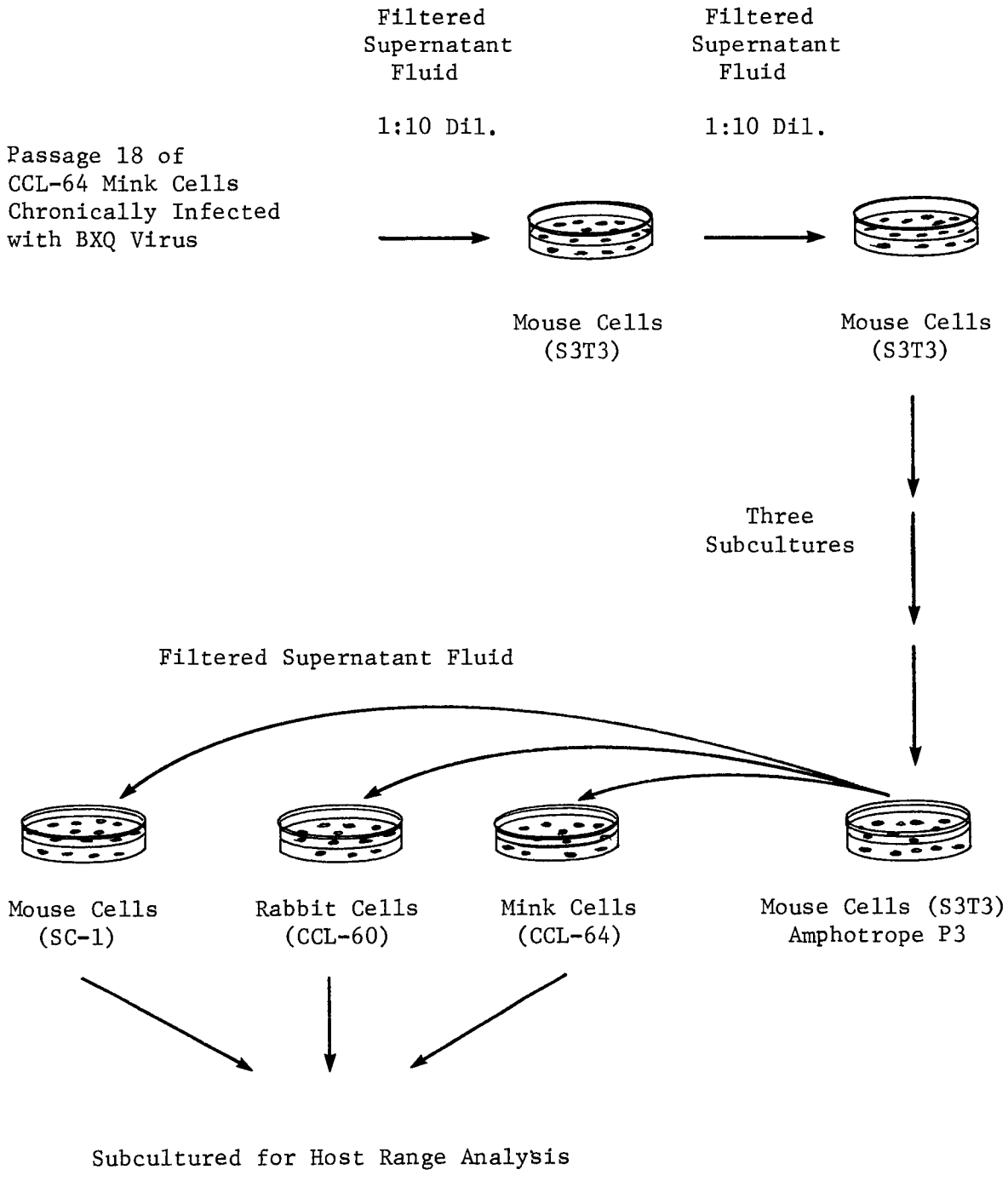


Figure 1. Scheme for Isolation of Amphotropic Virus

TABLE 6

Host Range Assay of Amphotropic Virus

Cells	Immunofluorescent Cells		
	SC-1	CCL-60	CCL-64
Mouse (SME)	33	10	21
Mouse (BALB/c)	12	0	4
Mouse (SC-1)	78	41	71
Rabbit (CCL-60)	23	63	79
Human (A_{204})	12	>1	7
Baboon	8	NT[a]	36
Quail	0	NT	27

[a]Not tested.

and the viral host range was assayed periodically. The results, shown in Table 7, indicate that upon prolonged subculture the virus began to lose its ability to infect heterologous cells. Virus from passage three of S3T3 cells would infect 9% of the rabbit CCL-60 cells while virus from passage eight would infect less than 1% of the same cells. Filtered virus from passage eight of S3T3 cells was used to infect CCL-64 cells, and 10% of these mink cells were immunofluorescent positive after 48 hours. When these cells were subcultured until all cells were expressing virus (10 passages), the supernatant fluid was assayed for host range (Table 7, last column) and the results indicate that the virus had lost its ability to infect N- and B-type mouse cells.

The above experiments indicate a selective host pressure against the amphotropic virus when passaged in the mouse S3T3 cells. However, when the mink CCL-64 cells used in the isolation procedure (Fig. 1) were infected prior to this selection, the virus maintained its amphotropic characteristic through 13 subcultures of the mink cells, with 52% of the mouse S3T3 cells and 90% of the rabbit CCL-60 cells being infected by the virus.

Inoculation of Cells Infected with Virus Isolates into Mice

To test for the potential tumorigenicity of the amphotropic and xenotropic viruses, mink CCL-64 cells were infected with medium from the BXQ cells, a mixture of amphotropic and xenotropic viruses, and BALB/c clone A31 cells were infected with amphotropic virus from S3T3 P3 cells. When all cells were expressing virus, detected by immunofluorescence, they were inoculated into newborn BALB/c nude (athymic) mice. Groups of five mice each were inoculated as follows:

TABLE 7

Amphotropic Virus Host Range Pattern After Passage in S3T3 and Mink Cells

Cells	Immunofluorescent Cells (%)		
	S3T3 P3[a]	S3T3 P8[a]	CCL-64 P10[b]
Mouse (SME)	31	51	0
Mouse (BALB/c)	4	2	0
Mouse (SC-1)	73	83	32
Rabbit (CCL-60)	6	1	71
Human (A_{204})	1	1	2

[a]Virus from S3T3 cells after the number of subcultures indicated was assayed on the cells shown.

[b]Virus from S3T3-P8 was used to infect CCL-64 cells. After 10 subcultures, virus was assayed as indicated.

1. One group received 10^6 mink CCL-64 cells which contained the amphotropic and xenotropic viruses.
2. A second group received 5×10^6 BALB/c clone A31 cells containing the amphotropic isolates.
3. Five mice each received either normal mink CCL-64 cells or uninduced BALB/c clone A31 cells.

All of the animals were examined once a week for the development of tumors. After six months of observation none of the mice have died or shown any tumor development or leukemia.

CHAPTER IV

DISCUSSION

Evidence for the genetic transmission of murine leukemia virus was provided by the discovery that mouse cells in culture could spontaneously release RNA type C virus after growth for many generations in the absence of detectable virus production (Rowe et al., 1971; Rowe, 1972; Rowe and Hartley, 1972). With the discovery of highly efficient chemical inducers it became increasingly clear that endogenous type C viruses could be activated to replicate from an unexpressed state from almost every mouse cell culture tested (Lowy et al., 1971; Aaronson and Dunn, 1974).

Hybridization studies with type C viral DNA probes to normal cell DNA lead to the discovery that the high molecular weight DNA of many mouse cells, including the BALB/c strain, contained multiple copies (9-13) of type C virus specific information (Gelb et al., 1973; Chattopadhyay et al., 1974). These studies provided confirmation for the observations by Rowe and Hartley (1972) that the type C viruses are genetically transmitted within the mouse cell DNA.

The endogenous type C viruses can be efficiently induced by treatment with the halogenated pyrimidines 5-iodo-2'-deoxyuridine (IdU) and 5-bromo-2'-deoxyuridine (BrdU). Evidence that IdU incorporation into the cellular DNA is required for virus induction comes from studies by Teich et al. (1973) and Ihle et al. (1974) using the AKR cell line. Cytosine arabinoside, excess thymidine, or serum depletion which block DNA synthesis are capable of decreasing induction, whereas cells exposed to suboptimal concentrations of halogenated pyrimidines can be induced by exposure to white light (Teich et al., 1973).

When the BALB/3T3 clone A31 cell line was treated with 100 μg/ml of IdU for 24 hours only a small percentage of the cells survived. This cytotoxicity may have been due to the incorporation of more of the chemical into the DNA of A31 cells than in other mouse cell lines tested in our laboratory. Because these cells contain multiple copies of at least two endogenous MuLVs, which are activated by IdU, the possibility exists for multiple induction events at more than one site in the cellular DNA.

The original intention of the experiment was to transfer the BXQ virus supernatant fluid to rabbit cells to produce a xenotropic virus pool. Therefore the early passages of the cells were not extensively examined by immunofluorescence and XC plaque assays for the presence of an N-tropic virus. If one did exist there is the possibility that it was lost due to the Fv-1 gene restriction against its spread to other cells.

When later passages of the BXQ cells proved to be tumorogenic it was of obvious importance to isolate the virus(es) being produced by the cells to determine if a sarcomagenic virus had been induced. Examination of the BXQ virus(es) showed infection of a broad host range of cells but no syncytial plaques in the XC test. If the ability to infect all mouse cells was the result of a mouse-tropic, probably N-type, non-plaquing virus, then by infecting mink cells and passaging these filtered culture fluids to other mink cells, the chance of a mouse-tropic virus infecting the mink cells would be unlikely. This procedure should also eliminate a phenotypic mixture of virus as described by Bassin et al. (1975). Phenotypic mixtures have always demonstrated a dual sensitivity to restriction and dissociate after passage in mouse cells (Rein et al., 1976). However, the BXQ virus maintained in mink cells was stable in

its infectivity pattern of mouse cells beyond 20 mink cell passages, indicating that a phenotypic mixture of viruses was not the reason for its broad host range characteristics.

Purification by limit dilution yielded the BALB:virus-2 (xenotropic virus), but revealed another virus to be present in almost equivalent titer. Once the xenotropic virus was isolated the BXQ virus mixture was taken through several passages in mouse S3T3 cells which eliminated the xenotropic virus, and as shown in Fig. 1, p. 20, was inoculated onto SC-1 mouse, rabbit, and mink cells for host range characterization.

The amphotropic type virus which was isolated may have been activated through the derepression of an endogenous sequence coding for a specific broad host range virus. This virus would be of the same type as that isolated from feral mouse embryos as described by Hartley and Rowe (1976). Another possibility is the recombination of the genetic material of the two endogenous viruses before or during transcription. This possibility would be difficult to prove with our current limited knowledge available on mouse chromosomes and/or endogenous virus genomes contained in them. During the initial amphotropic virus isolation, serial passage in S3T3 cells had an effect on the ability of the virus to infect heterologous cells. This decrease in infectivity did not prevent the virus from growing to high titer in mink cells, however, this mink cell adapted virus would no longer infect mouse cells.

A model for a genotypic mixture of N-tropic and xenotropic viruses seems to fit the host range pattern of amphotropic virus and its inability to trigger the XC plaque response. If the N-tropic and xenotropic viral genomes were aligned in tandem on the A31 cell DNA, then the

treatment with a cytotoxic dose of IdU might allow the analogue to be incorporated into both of these regions, and could have led to consecutive transcription of the two viral genomes instead of separate transcription of each set of sequences. Evidence to support a tandem arrangement for viral genomes comes from experiments by Shoyab et al. (1976). When chicken cells were infected with avian RNA type C virus, the provirus was integrated in or adjacent to the regions of endogenous viral information. Because BALB/c cells contain multiple copies (9-13) of the genetic information for at least two different endogenous viruses it would seem likely that the N-tropic and xenotropic genomes could lie adjacent to each other in the cellular DNA.

When RNA type C viruses infect a permissive cell the viral enzyme RNA dependent DNA polymerase (reverse transcriptase) transcribes a DNA copy of the RNA genome. The DNA copy is believed to be integrated into the cellular DNA from which progeny RNA type C viruses are transcribed (Temin and Baltimore, 1972). During the process of infection in the N-tropic S3T3 cell the genotypically mixed, amphotropic virus genome could be transcribed and a DNA conscript integrated into the cellular DNA. There should be no host cell pressure or restriction against the N-tropic segment of the viral genome as may occur for the xenotropic segment (Aaronson and Stephenson, 1973). Therefore, during serial passage more N-tropic virus would be available to infect the S3T3 cells, eventually leading to an almost pure N-tropic virus population, since the xenotropic virus would be unable to exogenously infect S3T3 cells.

The SC-1 mouse, rabbit, and mink cells lack the Fv-1 gene for mouse-tropic virus restriction and cellular restriction for xenotropic viruses

(Hartley and Rowe, 1975). Therefore the amphotropic virus should not be subjected to the host pressures which occurred in the FV-1 gene containing mouse cells. The amphotropic and xenotropic viruses, once integrated into the cellular genomes should be able to replicate in these cells without restriction.

Although little is known of the factors responsible for syncytial cell formation in the XC plaque test the lack of a response by xenotropic virus in the XC plaque assay could occur if the inability to respond is linked to the lack of a viral coat molecule. The amphotropic virus coat could presumably contain an equal distribution of N-tropic and xenotropic coat molecules. If there is a critical number of molecules necessary for syncytial formation upon contact with an XC cell then the lack of sufficient quantities of this molecule would abrogate the ability to form syncytia.

The theories for the origin of an amphotropic virus are purely conjecture. The answer may be forthcoming with the application of biochemical probes for the viral genome to determine the origin of its components.

Normal A31 and mink cells were infected with purified amphotropic and xenotropic virus respectively. After several passages, virus producing cells were inoculated into newborn BALB/c athymic nude mice. After six months none of the mice developed tumors nor did they show signs of developing leukemia. Therefore, the treatment of A31 cells by IdU may have led to simultaneous transformation and induction of endogenous viruses, but the two events may have been independent of each other.

Because the amphotropic virus did not trigger the XC cell response the virus would have been missed if the infected cells were not also

examined by the immunofluorescence assay. The induction of endogenous viruses by chemicals may lead to other recombinants or genotypic mixtures. Therefore it is essential that all methods of assaying RNA type C viruses be employed to either eliminate possible virus contaminants or detect new viral recombinants.

In summary, the transformed BXQ cells were found to produce two viruses which have been isolated and characterized as to their host range specificity. The xenotropic virus has been found before, but not isolated for routine laboratory use; the other an amphotropic virus, with a broad host range, was isolated and may be the result of a recombinational event between endogenous N-tropic and xenotropic virus or a stable genotypic mixture of these two viruses. Isolation procedures using selective host pressure were employed to isolate and purify the two viruses so that their complete host ranges could be studied.

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