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Transfer of *Fv-1* Locus-Specific Resistance to Murine N-Tropic and B-Tropic Retroviruses by Cytoplasmic RNA

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A standardized bioassay for transfer of *Fv-1* gene-specific resistance to N-tropic and B-tropic murine retroviruses was developed using XC plaque reduction in SC-1 (*Fv-1*$^-$) cells inoculated with virus. Testing of subcellular fractions of restrictive cells showed that the resistance transfer activity was present in the cytoplasmic (microsomal and cytosol) fractions. The activity of the cytoplasmic extract was destroyed by treatment with ribonuclease, but not with deoxyribonuclease or proteases. RNA prepared by phenol-chloroform extraction of mouse tissues, including embryos and livers of weanling mice, transferred *Fv-1* locus-specific resistance into DEAE-dextran-treated SC-1 cells. The activity of isolated RNA preparations against virus of the appropriate host-range type has been demonstrated to correspond to the *Fv-1* genotypes of the cell sources. The specific transfer of resistance with cellular RNA was effective within a 5- to 6-h period from 2 h before to 4 to 5 h after virus infection. Sucrose gradient centrifugation of the RNA showed that the activity sedimented as a broad peak, with an apparent maximum in the 22S region. Affinity chromatography of whole-cell RNA on polyuridylic acid-Sepharose tended to separate more activity into the polyadenylic acid RNA fraction than the non-polyadenylic acid RNA fraction. Except for the reciprocal inhibitory activity for the two host-range virus types, the RNAs of *Fv-1*^{n} and *Fv-1*^{b} specificities showed similar properties in all aspects studied.

The importance of the *Fv-1* gene locus in determining host resistance of mouse cells to infection with N- and B-tropic murine retroviruses has been experimentally established in animal experiments (1, 24, 27, 33) as well as in cell culture systems (15, 32). The *Fv-1* locus may be represented by at least two alleles, *Fv-1*^{n} and *Fv-1*^{b}, which are expressed by all strains of laboratory mice (see review in ref. 25). Cells of the *Fv-1*^{n} genotype are resistant to B-tropic viruses but are susceptible to N-tropic viruses, whereas cells of *Fv-1*^{b} genotype show reciprocal resistance and susceptibility; heterozygotic cells (*Fv-1*^{nb}) are resistant to both N- and B-tropic viruses. The dominant expression of the *Fv-1* locus appears to be directed at a specific virion component (26, 35), the identity of which is yet to be determined.

Studies on the virus replication cycle have established the following facts. The inhibition of virus infection by *Fv-1* gene action occurs intracellularly after membrane adsorption and penetration of virions (18, 22). Studies by molecular hybridization have shown that whereas proviral DNA is formed in similar quantity in resistant and permissive cells after virus inoculation, it does not appear to undergo integration in resistant cells (19, 41). Recent DNA transfection studies have shown that infectious DNAs of N- and B-tropic viruses, unlike infectious virions, are not restricted by the *Fv-1* gene (16, 17). Further, unintegrated proviral DNA preparations from *Fv-1* permissive cells are infectious even in non-permissive cells, whereas similar preparations from *Fv-1* restrictive cells are usually incapable of transfection (Yang, Boone, Hsu, Yang, Tennant, and Brown, manuscript in preparation). These results, therefore, suggest that the *Fv-1* gene restriction involves the synthesis of proviral DNA which may be defective for integration into the cell genome.

The *Fv-1* gene is located on mouse chromosome 4 (38), but the chemical nature of the gene product or the cellular function which presumably interferes with competent proviral DNA synthesis in restrictive cells has not been defined. Previous studies demonstrated that specific resistance could be transferred to permissive cells by use of cell-free extracts (44). Genetic analysis of the resistance transfer has provided

evidence that the effect is specific for the appropriate host-range type of virus and the *Fv-1* genotype of cells used for preparation of the extracts (43). These results suggest that resistant cells constitutively synthesize a product which restricts competent proviral DNA formation. The studies described here indicate that the resistance transfer of *Fv-1* phenotypes is mediated by RNA molecules extractable from the cytoplasm of restrictive cells.

MATERIALS AND METHODS

Viruses. N-tropic (Gross strain), B-tropic (WN1802B), and NB-tropic (Moloney strain) murine leukemia viruses were obtained originally from W. P. Rowe and Janet Hartley of the National Institutes of Health, Bethesda, Md. N-tropic virus was prepared by infecting secondary cultures of NIH Swiss mouse embryo cells with the original stock of virus and then collecting the culture medium of the infected cells at 18-h intervals; the B-tropic virus was prepared similarly in secondary cultures of BALB/c mouse embryo cells. The pooled medium containing the virus was centrifuged to remove cell debris at 5,000 rpm for 30 min in a Sorvall SS-34 rotor, and the clarified medium was frozen in 1-ml aliquots and stored at −170°C. The host-range specificity of each virus stock was verified by titration on BALB/c and NIH Swiss mouse embryo cells. The virus stocks used in the present study were all within three passages of the original parent stocks, and they were free of mycoplasma or xenotropic murine retroviruses. The absence of xenotropic virus was determined by immunofluorescence assay in cultures of mink (CCL64) cells and rabbit (CCL60) cells inoculated with the virus stock.

Cell cultures. Primary cell cultures were prepared from embryos of *Fv-1*^{n} NIH Swiss mice (Microbiological Associates, Inc., Walkersville, Md.) and *Fv-1*^{b} BALB/c mice (Cumberland View Farm, Clinton, Tenn.). The embryo cell cultures were maintained in Eagle minimum essential medium containing 10% fetal calf serum (Microbiological Associates, Inc., Bethesda, Md.), 2 mM glutamine, 100 μg of streptomycin per ml, and 100 U of penicillin per ml. SC-1 cells (*Fv-1*$^-$), which are permissive to both N- and B-tropic viruses, were obtained from Janet Hartley. The SC-1 cells were grown in McCoy 5A medium containing 5% fetal calf serum and supplemented as above.

Bioassay of *Fv-1*-specific gene products. Cell-free extracts and RNA were tested for their ability to transfer specific *Fv-1* locus resistance properties into susceptible cells, essentially as previously described (43, 44). Because of the complexity of the system for assaying the *Fv-1*-specific RNA, and also to obtain a high degree of reproducibility, stringent control was maintained over the many variables of the assay. Unless otherwise stated, the following are the standard procedures of the assay: The SC-1 cells were used only between passages 80 and 96 and were used 3 to 4 days after subculturing, when they were subconfluent and actively dividing. They were trypsinized and plated at 8.0×10^4 to 9.0×10^4 cells per 35-mm well (Linbro Chemical Co., New Haven, Conn.). After 18 h, the cells were treated with DEAE-dextran (Pharmacia Co., Piscataway, N.J.) at 25 μg/ml in serum-free McCoy medium for 30 min at 37°C. The cells were then washed once with 2 ml of the same medium and inoculated with 2 ml of the same medium containing an appropriate dilution of virus (multiplicity of infection [MOI] = 0.001). After incubation at 37°C for 2 h (or for 15 min in some experiments), the virus-containing medium was removed, and the cells were again washed with serum-free McCoy medium. The cell extract or RNA to be tested was applied directly and uniformly onto the cells in the well in a volume of 0.2 ml of serum-free McCoy medium. After brief mixing, 0.8 ml of the same medium was added, and the cells were incubated for another 2 h. At the end of the incubation, the RNA-containing medium was replaced by 2 ml of complete McCoy medium. Three days later, the XC plaque assay (39) was performed by irradiating the SC-1 cells with UV light (150 J/m^2) and overlaying 4×10^5 XC cells in each 35-mm well. Two more days later, the cell cultures were fixed and stained, and the syncytial plaques were counted.

Where indirect immunofluorescence was used to assay virus infectivity, glass cover slips were placed in the 35-mm wells before cell plating, and the virus was inoculated at an input MOI of 0.2 to 0.5 (PFU per SC-1 cell). The cells were washed three times with serum-free McCoy medium before the RNA was added. The cover slips were harvested 42 to 48 h after virus infection and RNA treatment, fixed in cold acetone for 30 min, air dried, and stained as previously described (42).

Preparation and fractionation of cell homogenate. Crude cell extracts of secondary mouse embryo cell cultures were prepared by sonic disruption as previously described (43, 44). However, for the purpose of subcellular localization of *Fv-1*-specific activity, cell homogenates were made under aseptic conditions by the following procedure. Secondary mouse embryo cells, grown as a monolayer in roller-bottle cultures, were rinsed three times with Hanks balanced salt solution, scraped off with a windshield wiper (Volkswagen), and washed twice in cold phosphate-buffered saline by centrifugation in a refrigerated centrifuge ($600 \times g$, 10 min). All subsequent procedures were carried out at 0 to 4°C. The washed cells of 1 to 2-ml packed volume were first suspended in 2 ml of phosphate-buffered saline and then mixed with 4 ml of a hypotonic solution containing 10 mM Tris-chloride (pH 7.5) and 3 mM $MgCl_2$. The swollen cells were collected by centrifugation and then mixed with 4 ml of the hypotonic solution in a Dounce homogenizer (12 strokes). The homogenate was immediately mixed with 1 ml of 5× Hanks balanced salt solution containing 25% glycerol. Differential centrifugation of the homogenate was performed at $600\times g$ for 10 min, $7,000 \times g$ for 15 min, and $150,000 \times g$ for 60 min to obtain "nuclear," "mitochondrial," "microsomal," and "supernatant" fractions. The pellet fractions were suspended in 10 ml of Earle balanced salt solution by the use of a loose-pestle homogenizer; the supernatant fraction was diluted with 5 ml of the Earle salt solution. The subcellular fractions of the homogenate were immediately assayed for activity.

For testing the enzyme inactivation of the *Fv-1*-specific activity of the cell extracts, homogenization of

secondary embryo cells was performed as described above, but the supernatants were obtained by centrifugation at 100,000 × *g* for 15 min. Five aliquots of the supernatant were adjusted to contain either 10 μg of deoxyribonuclease (DNase I, ribonuclease-free, from Worthington Biochemicals, Freehold, N.J.) per ml; 10 μg of pancreatic ribonuclease A (5× crystallized, Calbiochem, La Jolla, Calif.) per ml, 20 μg of trypsin (3× crystallized, Sigma Biochemical Co., St. Louis, Mo.); 25 μg of self-digested Pronase (Calbiochem) per ml; or no enzyme. After incubation at 37°C for 10 min, the treated cell extracts were adjusted to contain 3 mg of bovine serum albumin per ml and used directly in the assay.

Extraction of RNA. Preparations were made from cells of secondary mouse embryo cell cultures, from whole embryos of 12 to 14 days' gestation, and from livers of 6- to 8-week-old mice. The cultured cells were harvested and broken as described above, except that after hypotonic homogenization 0.25 volume of a solution containing 1.25 M sucrose, 50 mM Tris-chloride (pH 7.6), 0.5 M KCl, and 5 mM EDTA was added to the homogenate. Embryos or livers were weighed, minced, and homogenized with 5 volumes (vol/wt) of an ice-chilled medium containing 0.25 M sucrose, 5 mM Tris-chloride (pH 7.6), 100 mM KCl, and 1 mM EDTA. The homogenate was spun in a refrigerated centrifuge at 8,000 × *g* for 15 min, and the RNA was extracted from the supernatant according to the phenol-chloroform procedure of Perry et al. (31), modified as follows. To the supernatant was added 0.05 volume of 10% sodium dodecyl sulfate, 1 volume of water-saturated phenol redistilled from fused crystal phenol (Matheson, Coleman and Bell Co., Norwood, Ohio), and 1 volume of chloroform. The mixture was shaken for 5 min and then centrifuged at 6,000 × *g* for 15 min. The aqueous phase was removed and saved. The interphase layer was removed, its volume was measured, and it was further extracted with 0.7 volume of 0.1 M Tris-chloride (pH 7.6) and 1.3 volumes of chloroform. The two aqueous-phase solutions were combined and extracted sequentially with 1 volume of phenol-chloroform (1:1) and then with 1 volume of phenol alone. After extraction, the aqueous phase was mixed with 0.1 volume of 20% potassium acetate (pH 5.6) and 2.5 volumes of 95% ethanol (−20°C), and the mixture was left standing overnight at −20°C. The RNA was pelleted at 10,000 × *g* for 10 min, dissolved in distilled water (2 volumes per original wet tissue weight), and re-precipitated overnight from 68% ethanol at −20°C. The RNA was again collected and dissolved for spectrophotometric measurement at 260 nm and 280 nm. By assuming that 20 absorbancy units at 260 nm equals 1 mg, the yield of RNA was 3 to 5 mg/g of wet-tissue weight. The RNA was stored as a suspension of 0.2 mg/ml in 68% ethanol at −20°C. Throughout the preparation procedure, strict precaution was taken to prevent ribonuclease contamination; all glassware used was washed with 1% diethylpyrocarbonate, rinsed with distilled water, and autoclaved. Some lots of RNA prepared and stored in this manner have retained specific inhibitory activity when tested for 10 months.

Velocity sedimentation. RNA was sedimented in 10 to 30% (wt/wt) linear sucrose gradients, containing 10 mM Tris-chloride (pH 7.6), 10 mM NaCl, and 1 mM EDTA, at 25,000 rpm for 22 h in a Beckman SW27 rotor. RNA was monitored using an ISCO gradient fractionator and was separated into fractions corresponding to 4S, 18S, and 28S RNA as well as material sedimenting in the 4 to 18S, 18 to 28S, and greater than 28S regions. RNA in the fractions was precipitated from ethanol, dissolved in phosphate-buffered saline, and tested for resistance transfer activity. Active fractions were pooled and then sedimented in a sucrose gradient (10 to 30%) at 40,000 rpm for 8 h in a Beckman SW41 rotor. Fractions of the gradient were again collected and tested for activity.

Poly(U)-Sepharose chromatography. RNA was fractionated according to polyadenylic acid [poly(A)] composition by affinity chromatography on a polyuridylic acid [poly(U)]-Sepharose column. Poly(U)-Sepharose (5 to 8 mg/g, from Pharmacia Co.) was suspended in 0.5% sodium dodecyl sulfate. A water-jacketed column with an inner diameter of 0.9 cm, maintained at 37°C, was packed with about 3 ml of the swollen gel, which was subsequently equilibrated with a solution containing 10 mM Tris-chloride (pH 7.5)–0.5 M NaCl–0.5% sodium dodecyl sulfate. RNA dissolved in water was heated at 65°C for 5 min, quickly chilled on ice, adjusted to contain the same buffer and salts as the column equilibration solution, and applied at 37°C to the column. Effluent from the column was monitored by absorbance at 254 nm, and the column was washed with the equilibration buffer to collect non-poly(A) RNA. The column was subsequently eluted with a solution of 0.1 M NaCl, 0.01 M Tris-chloride (pH 7.5), and 0.1% sodium dodecyl sulfate. When no more RNA could be eluted, the temperature of the column was raised to 50°C, and poly(A) RNA was eluted with distilled water at that temperature. The RNA from each elution was precipitated twice from 68% ethanol to remove sodium dodecyl sulfate, dissolved in phosphate-buffered saline, and tested for resistance transfer activity.

RESULTS

Subcellular localization. We previously reported the transfer of *Fv-1* gene resistance properties using cell-free extracts prepared by mild sonic disruption of cultured embryo cells (43, 44). In our experience, inhibition of 20% or less may represent nonspecific activity, and only those fractions demonstrating greater inhibition were considered active; they were considered specific only if the inhibitory activity was directed against the appropriate virus. To study the subcellular distribution of this activity, we employed cell homogenization in hypotonic solution with subsequent differential centrifugation. This homogenization procedure was found to yield active preparations; hence, four subcellular fractions of BALB/c mouse embryo cells were obtained for testing transfer of resistance against N-tropic virus (Table 1). The activity was detected in the microsomal and postmicrosomal supernatant fractions but not in the nu-

clear or mitochondrial fractions. Although the result could reflect differences in uptake of the active molecules from the respective fractions rather than true subcellular distribution, the soluble form of the activity made it suitable for further characterization. The specific activities of *Fv-1*^{n} and *Fv-1*^{b} cell supernatants were in the excluded volume following gel filtration on Sephadex G25 and were eluted in a diffuse pattern from DEAE-cellulose and from phosphocellulose; an attempt to extract the microsomal fraction with 1 M KCl solution resulted in complete loss of activity (data not shown).

Ribonuclease sensitivity. To determine the biochemical nature of the active components in the cell extract, we subjected the extracts to digestion with hydrolytic enzymes (Table 2). The ability of cell extracts to transfer *Fv-1* gene-specific resistance was found to be destroyed selectively by pancreatic ribonuclease but not by trypsin or deoxyribonuclease; Pronase was used in later experiments but showed no effect on the inhibitory activity. The same result was obtained upon digestion of the cytoplasmic fraction of secondary embryo cell cultures as well as with extracts of the livers from weanling mice of both *Fv-1* genotypes (data not shown). This indicated that the active component(s) might be RNA or ribonucleoprotein.

TABLE 1. *Transfer of resistance with subcellular fractions of Fv-1*^{b} *cells*[a]

Treatment with fraction:	% SC-1 cells infected with:		
	B virus	N virus	NB virus
Total homogenate	26 ± 8 (0)	8 ± 1 (62)	22 ± 3 (12)
Nuclear	21 ± 5 (5)	19 ± 4 (9)	25 ± 4 (0)
Mitochondrial	20 ± 4 (9)	20 ± 5 (4)	20 ± 3 (20)
Microsomal	24 ± 2 (0)	11 ± 2 (48)	23 ± 4 (8)
Supernatant	22 ± 8 (0)	5 ± 2 (76)	24 ± 6 (4)
No treatment	22 ± 6	21 ± 3	25 ± 8

[a] SC-1 cells, grown on cover slips in 35-mm wells, were treated sequentially with DEAE-dextran for 30 min, with the virus at MOI of 0.2 (XC PFU per SC-1 cell) for 2 h, and with subcellular fractions (prepared as described in the text) for 2 h. At 46 h after initial virus exposure, the percentage of infected cells was determined by immunofluorescence assay (42). The data are averages of countings of 10 fields (50 to 100 cells per field) and standard errors of the mean. Numbers in parentheses indicate percentage of inhibition relative to "no treatment" control.

Activity of RNA isolated by phenol extraction. Postmitochondrial supernatants of mouse cells and tissues were extracted with phenol sequentially at pH 7.6 and at pH 9.0, according to a published procedure (4), to obtain two RNA fractions relatively poor and rich, respectively, in mRNA activities. The DEAE dextran-pretreated secondary cultures of mouse embryos were incubated for 2 h with the appropriate virus types and then with the RNA preparations for another 2 h; 48 h later, the cultures were fixed and examined for percentage of infected cells by immunofluorescence. Infectivity of B-tropic virus was inhibited by RNA derived from *Fv-1*^{n} NIH Swiss mice (20 to 60%); infectivity of N-tropic virus was inhibited by RNA derived from *Fv-1*^{b} BALB/c mice (35 to 50%); infectivity of NB-tropic virus was minimally affected by these RNA preparations (Table 3). Similar results were obtained in experiments using low MOI and the XC plaque assay (data not shown). The pH 9.0 RNA fraction appeared to be more active than the pH 7.6 RNA fraction on the basis of minimal effective dose, although the latter still contained considerable activity. According to the report of Perry et al. (31), more poly(A) RNA can be recovered from cellular ribosomes by extraction with a mixture of phenol and chloroform than by extraction with phenol alone. We found that the phenol-chloroform extraction method also gave a better yield of *Fv-1* resistance transfer activity than the extraction by phenol at the two pH's combined. Hence, the phenol-chloroform extraction of RNA was used in subsequent studies.

Factors affecting assay of *Fv-1* gene-specific RNA. Different kinds of technical difficulties were encountered when RNA preparations instead of crude cell extracts were used to trans-

TABLE 2. *Effects of enzyme treatment on resistance transfer activity of Fv-1*^{b} *and Fv-1*^{n} *cell extracts in SC-1 cells*[a]

Treatment	% Cells infected			
	Fv-1^b extract		*Fv-1*^{n} extract	
	N virus	B virus	N virus	B virus
None	12 ± 3 (55)	51 ± 7 (1)	29 ± 3 (0)	20 ± 3 (61)
Deoxyribonuclease	14 ± 2 (51)	52 ± 4 (0)	28 ± 3 (0)	24 ± 5 (54)
Trypsin	13 ± 2 (54)	46 ± 7 (9)	29 ± 5 (0)	25 ± 4 (52)
Ribonuclease	26 ± 6 (6)	46 ± 6 (9)	24 ± 3 (13)	45 ± 8 (13)
No extract	28 ± 4	52 ± 7	28 ± 4	52 ± 7

[a] Conditions of assay and data presentation are the same as described in Table 1, footnote *a*, except that SC-1 cells were fixed for immunofluorescence measurement at 48 h after virus exposure.

TABLE 3. *Inhibitory activity of mouse cell RNA preparations against virus infection*[a]

Source and amount of RNA for treatment	B virus		N virus		NB virus	
	B-ME	S-ME	B-ME	S-ME	B-ME	S-ME
None	51 ± 4		5 ± 1		35 ± 4	
Fv-1^n embryo (pH 7.6) 30 μg	26 ± 3 (49)		14 ± 2 (0)		33 ± 4 (6)	
Fv-1^n embryo (pH 9.0) 5 μg	17 ± 3 (67)		11 ± 2 (0)		33 ± 3 (6)	
None		7 ± 2		48 ± 6		40 ± 9
Fv-1^b embryo (pH 7.6) 30 μg		7 ± 2 (0)		28 ± 6 (42)		36 ± 7 (10)
Fv-1^b embryo (pH 9.0) 5 μg		6 ± 2 (14)		25 ± 4 (48)		52 ± 11 (0)
None	83 ± 3				45 ± 5	
Fv-1^n liver (pH 7.6) 12 μg	66 ± 6 (20)				50 ± 4 (0)	
Fv-1^n liver (pH 9.0) 4 μg	39 ± 4 (53)				42 ± 3 (6)	
None				37 ± 3		47 ± 11
Fv-1^b liver (pH 7.6) 12 μg				24 ± 2 (35)		48 ± 7 (0)
Fv-1^b liver (pH 9.0) 4 μg				19 ± 3 (49)		57 ± 6 (0)

[a] Secondary cell cultures of BALB/c mouse embryos (B-ME) or NIH Swiss mouse embryos (S-ME) were exposed to the virus at MOI of 0.2 to 0.5 (based on XC PFU titer in SC-1 cells) and then treated with indicated doses of pH 7.6 RNA fraction or pH 9.0 RNA fractions extracted from embryos or livers of weanling mice. Differential-pH phenol extraction of RNA followed essentially the procedures of Brawerman et al. (4). Immunofluorescence assay was performed at 56 h after virus exposure. For details of data presentation, see Table 1, footnote *a*.

fer the *Fv-1* gene-specific resistance properties. The crude cell extracts were unstable, and the control of sonic disruption was important to obtain active preparations. In contrast, the RNA preparations were generally stable, but difficulty was encountered in the cell assay system. There are three major constraints associated with the cell assay system. (i) RNA can interfere with virus absorption, and this is particularly evident if RNA is added to the cells together with or immediately before virus administration. The uptake of [^{3}H]uridine-labeled virus by cultured mouse cells was inhibited up to 50% when RNA was given between DEAE-dextran treatment and virus inoculation. This was due presumably to neutralization of the charge effect of DEAE-dextran on the cell membrane, since another DEAE-dextran treatment immediately before virus inoculation eliminated this inhibition of virus absorption (data not shown). This non-*Fv-1*-related effect of RNA was also seen when RNA was mixed with virus before addition to the cells (Fig. 4). Therefore, in our assay, RNA was routinely inoculated following virus administration. (ii) Under some conditions RNA can stimulate cell growth, which may grossly affect virus infectivity. This was notable in secondary cultures derived from primary embryo cells more than 1 week old. Using cultures derived from logarithmically growing mouse cells avoids this complication. (iii) The extracted RNA molecules are in a form susceptible to ribonucleases which may be present on the plasma membrane of the cells (47). Thus, conditions under which RNA is presented to the cells are extremely critical. It was found that careful and complete removal of serum from cells, in combination with DEAE-dextran pretreatment, greatly enhanced the uptake and putative utilization of the inoculated RNA (46).

Reproducibility of the established bioassay of RNA. For the assay of resistance transfer activity, the XC plaque reduction method in SC-1 cells was more reproducible than assays in secondary embryo cells. Table 4 shows the results of XC plaque assays in SC-1 cells from more than 40 independent experiments performed over a 1-year period. A high degree of reproducibility has been obtained through stringent control of such factors as cell passage level, cell growth state, culture conditions, virus stock, and virus dilution.

The results of up to 17 independent resistance transfer experiments performed with three concentrations of a single preparation of RNA, extracted from livers of 6- to 8-week-old NIH Swiss *Fv-1*^{n} mice (pool 401), are shown in Fig. 1. Evidence of resistance transfer against B-tropic virus was apparent with this RNA preparation, and the level of B-tropic virus inhibition increased from 5 to 20 μg of RNA; there was negligible or no inhibition of N-tropic virus through several experiments. However, after 6 to 7 months of storage the level of nonspecific inhibition of N-tropic virus increased significantly. (Occurrence of the nonspecific inhibitory activity was often observed in RNA preparations stored frozen in phosphate-buffered saline in liquid N_2 freezer. This effect has been largely eliminated by storing RNA preparations as precipitates in 68% ethanol at −20°C) In similar experiments, specific resistance transfer against N-tropic virus was obtained with an RNA preparation from 6- to 8-week-old BALB/c (*Fv-1*^{b})

mouse livers (pool 430). The results of resistance transfer in early experiments with pools 401 and 430 show a statistically significant pattern of inhibition of the restricted virus (Fig. 2). Pool 430 (Fig. 2A) showed no significant plaque reduction at 5 μg, significant (≥40%) plaque reduction of N-tropic virus at doses of 10 and 20 μg, but some nonspecific inhibition of virus infectivity at 20 μg. Pool 401 (*Fv-1*^{n}) (Fig. 2B) showed, however, specific results at 5 (20%), 10 (36%), and 20 μg (43%) and only exhibited significant inhibition of the homologous host-range type of virus at doses ≥40 μg.

Other sources and extraction methods. In the search for more specific and inhibitory RNA preparations, we tested other mouse strains (such as C3H, C57BL, and RF mice) and also other RNA extraction methods. However, the NIH Swiss and BALB/c strains originally used continued to be the best sources. The phenol-chloroform deproteination of post-mitochondrial supernatant fluids yielded the most potent and specific preparations, while RNA prepared by a guanidinium chloride extraction procedure (5) has been consistently inactive or nonspecific, although RNA in these preparations appeared similarly intact by other physicochemical measurements. Other methods such as differential pH phenol extraction (4), extraction with a cresol-phenol-8-hydroxyguinoline mixture at elevated temperature (13), phenol extraction in combination with diethylpyrocarbonate (9), and $MgCl_2$ precipitation of ribonucleoprotein (30) followed by oligo(dT)-cellulose chromatography (23) did not yield preparations with greater specificity or degree of inhibition.

Mouse embryos (12 to 14 days) proved to be a better source of RNA than young adult livers. Pool 119 (Fig. 3A) prepared from *Fv-1* BALB/c embryos showed specific and statistically significant inhibition of N-tropic virus at a dose of 1 μg. Inhibition increased with increasing RNA dose up to 20 μg. Pool 217 (Fig. 3B) was even more inhibitory at 1 μg (40%), with some nonspecific inhibition at 20-μg doses. With these and other RNA preparations the maximal degree of specific inhibition was 50 to 60%.

TABLE 4. *Reproducibility of the XC plaque assay in SC-1 cells*[a]

Virus	Virus dilution ($-\log_{10}$)	No. of observations	Mean PFU	Standard deviation	Standard error
B	3.3	46	63.0	27.3	4.0
B	3.6	16	39.7	9.0	2.2
N	3.0	44	79.4	36.7	5.5
N	3.3	18	28.6	11.3	2.6

[a] PFU were determined between October 1976 and April 1977 in SC-1 cells using two virus stocks at an inoculum of 2.0 ml per 35-mm well. Cells were infected for 2 h following treatment with DEAE (25 μg/ml) for 1 h. XC cells were added 72 h postinfection at a concentration of 5.0×10^5 per 35-mm well and were fixed and stained 48 h later.

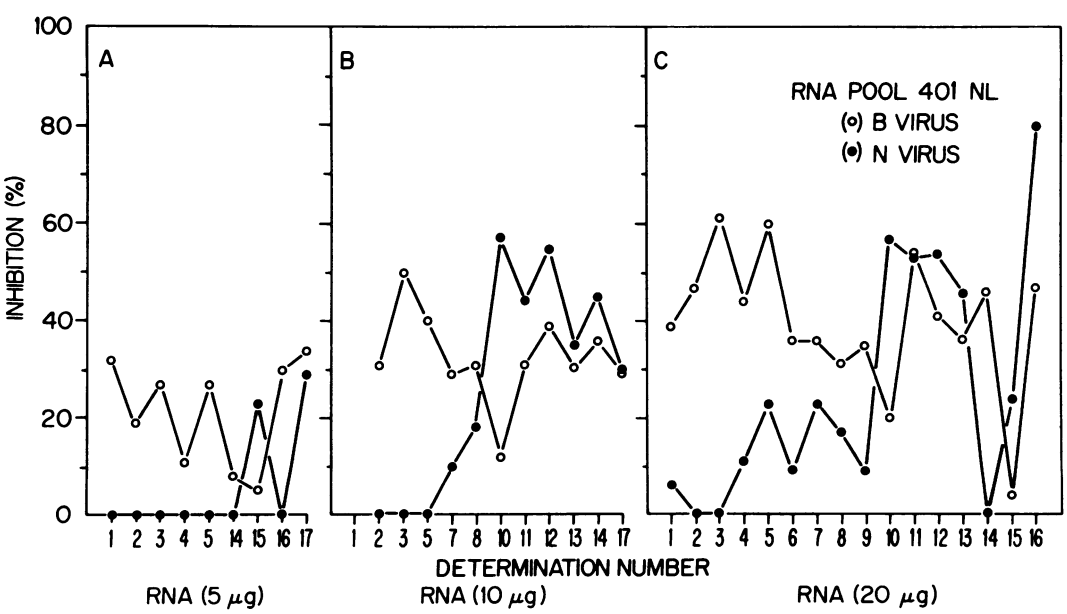

FIG. 1. *Transfer of resistance obtained over a 10-month period with RNA pool 401 NL (Fv-1*^{n}*) using the plaque reduction assay in SC-1 cells. Each point represents one determination, and the percentage of inhibition is plotted as a function of RNA concentration per 35-mm test well (8 × 10*^{4} *cells).*

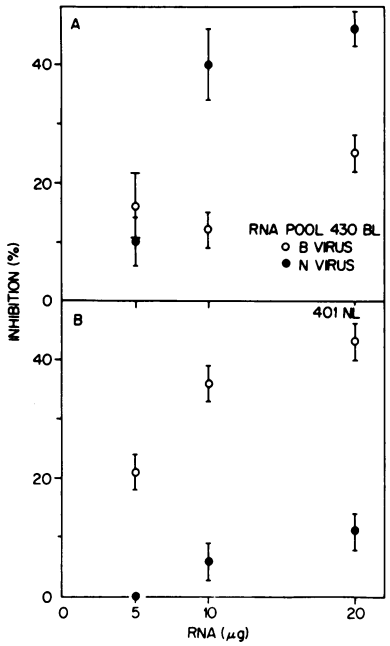

FIG. 2. *Transfer of resistance with mouse liver RNA assayed by plaque reduction in SC-1 cells over a 6-month period of time. (A) (RNA pool 430 BL, Fv-1^b) represents six independent observations at the 5-μg concentration and eight observations at the 10- and 20-μg concentrations. (B) (RNA pool 401 NL, Fv-1^n) represents six independent observations at the 5-μg concentration, five observations at 10 μg, and nine observations at 20 μg. In both (A) and (B), percentage of inhibition is plotted as a function of RNA concentration per 35-mm test well ± the standard error. The RNA pools are phenol-chloroform extractions from adult mouse livers.*

Time course of resistance transfer. Resistance transfer by RNA treatment was studied at various intervals before and after virus inoculation. To prevent nonspecific charge effects of polynucleotides, cells were treated with DEAE-dextran before RNA treatment and also before virus inoculation. Such experiments were performed with three active RNA preparations, two of *Fv-1*^{n} and one of *Fv-1*^{b}, with similar results. One such time course experiment on the inhibition of either N-tropic (nonspecific) or B-tropic (specific) virus in SC-1 cells treated with the pool 217 NE RNA preparation from NIH Swiss mouse embryos is shown in Fig. 4. At RNA doses of 5 and 10 μg per 35-mm well, a specific inhibitory effect on B-tropic virus was evident within the 6- to 7-h period from 2 h before to 4 to 5 h after virus inoculation. There was a marked nonspecific inhibitory effect on N-tropic virus only when the RNA preparation was given simultaneously with virus inoculation as discussed above, and this effect increased with increasing RNA dose. If cells were treated earlier than 2 h before or later than 6 h after virus infection, the RNA showed no apparent effect. This result is similar to that obtained by using the crude cell extract (44) and is consistent with the interpretation that the resistance-transferring molecules function at an early step in virus replication.

Size and poly(A) composition of the inhibitor RNA. The sedimentation pattern of active RNA preparations on sucrose gradients generally showed the three major peaks of 4S, 18S, and 28S. After centrifugation the sucrose gradient was separated into six fractions corresponding to the 4S, 4 to 18S, 18S, 18 to 28S, and greater than 28S regions for assay of resistance transfer activity. Figure 5 shows the results of the analysis of pool 217 NE (*Fv-1*^{n}) RNA. Pooled fractions of six separate gradients from the initial centrifugation in a Beckman SW27 rotor showed a diffuse pattern of activity (Fig. 5A). When the three fractions of 4 to 18S, 18S, and 18 to 28S RNA were combined and resedimented on another sucrose gradient in a Beck-

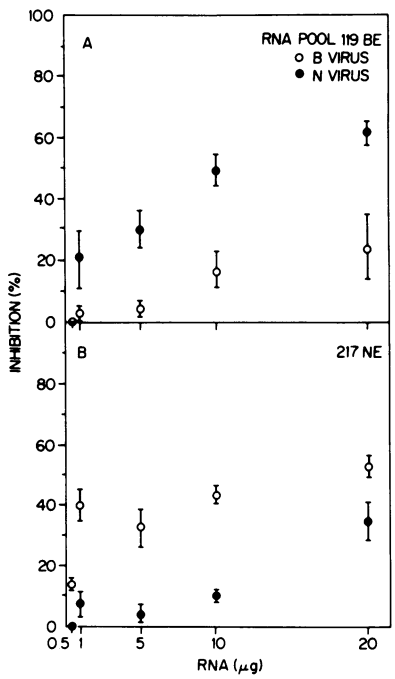

FIG. 3. *Transfer of resistance with mouse embryo RNA assayed by plaque reduction in SC-1 cells over a 2-month period of time. (A) (RNA pool 119 BE, Fv-1^b) represents seven independent observations, and (B) (RNA Pool 217 NE, Fv-1^n) represents five independent observations. In both (A) and (B), percentage of inhibition is plotted as a function of RNA concentration per 35-mm test well ± the standard error. The above RNA pools were prepared from phenol-chloroform-extracted 12- to 14-day embryos.*

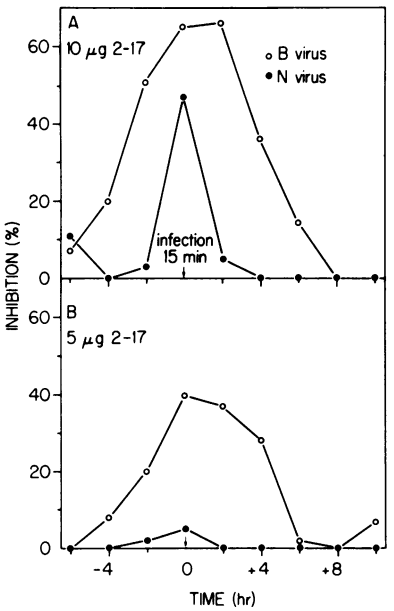

FIG. 4. *Time course study of the effect of FV-1^n 217 NE RNA treatment on the infectivity of B-tropic (specific) and N-tropic (nonspecific) viruses in SC-1 cells. SC-1 cells in 35-mm wells were pretreated with DEAE-dextran (25 μg/ml) for 20 min, washed, and incubated with 10 μg (A) or 5 μg (B) of pool 217 NE RNA (NIH Swiss embryo) for 2 h at the indicated times. All wells were infected with virus at 0 time for 15 min, and the XC plaque assay was performed 72 h after infection. The percentage of inhibition at each time point was determined from the averages of two RNA-treated and two sham-treated control wells.*

man SW41 rotor, maximal specific inhibitory activity for B-tropic virus was located in approximately the 22S region (Fig. 5B). Analyses of *Fv-1^b* RNA preparations revealed specific activity against N-tropic virus in a similar region. Pretreatment of RNA in 0.01 M NaCl solution at 85°C for 5 min before sucrose gradient sedimentation resulted in a slight decrease of the resistance transfer activity, but showed no apparent change in the broad sedimentation pattern. In a total of nine sucrose gradient sedimentation analyses with different lots of *Fv-1^n* and *Fv-1^b* RNA preparations, specific activity was generally detected in a broad size range with maximal levels in the 18 to 28S region. Compared to the embryo RNA preparations, the liver RNA preparations contained less specific activity below the 18S region.

Initial studies using the differential pH extraction procedure (4) showed higher activity of resistance transfer in the pH 9 RNA fraction than in the pH 7 RNA fraction, both on the basis of total extracted activity and effective minimal dose. This suggested that the active component is contained in RNA species other than tRNA's or rRNA's. Therefore, total RNA isolated by the phenol-chloroform procedure was chromatographed on poly(U)-Sepharose to separate poly(A) RNA from non-poly(A) RNA for the analysis. Table 5 shows the results with both an *Fv-1^n* Swiss mouse liver RNA preparation and a *Fv-1^b* BALB/c embryo RNA preparation. The respective inhibitory activity for the restricted virus tended to be segregated into the low-salt and water eluates, which represent mainly poly(A) RNA species. In some RNA preparations, especially those from mouse liver, some resistance transfer activity was also detected in the high-salt elute, which represents mainly the non-poly(A) RNA. However, the poly(A) RNA fraction consistently showed greater activity than the non-poly(A) RNA fraction.

DISCUSSION

In previous studies, we observed that the *Fv-1* gene-determined specific resistance against murine N- or B-tropic retroviruses could be transferred from restrictive mouse cells to per-

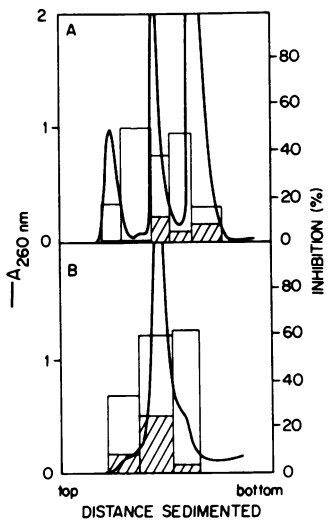

FIG. 5. *Fractionation of Fv-1 inhibitor activity by sucrose gradient velocity sedimentation. (A) RNA pool 217 NE (Fv-1^n) was sedimented on 10 to 30% (wt/vol) sucrose gradients at 25,000 rpm in a Beckman SW27 rotor for 22 h. Fractions were collected, precipitated with ethanol and redissolved in sodium phosphate-buffered saline, and 10 μg of each fraction was assayed for inhibitor activity by XC plaque reduction in SC-1 cells. (B) Fractions 2, 3, and 4 (10 μg of each) from (A) were pooled and centrifuged on linear sucrose gradients at 40,000 rpm in a Beckman SW41 rotor for 8 h. Open bars are percentage of inhibition of B-tropic virus, and lined bars are N-tropic virus. A_{260}, Absorbancy at 260 nm.*

TABLE 5. *Inhibitory activity of RNA fractions separated by poly(U)-Sepharose chromatography*[a]

Treatment	% Fluorescent cells		XC plaques per 35-mm well	
	B virus	N virus	B virus	N virus
NIH Swiss mouse liver RNA				
Unfractionated	26 ± 4 (41)	61 ± 3 (0)	63 (38)	117 (6)
Fractions eluted by				
0.5 M NaCl	35 ± 3 (20)	51 ± 3 (17)	72 (29)	108 (14)
0.1 M NaCl	23 ± 3 (47)	59 ± 4 (3)	65 (36)	105 (16)
Water	21 ± 2 (52)	62 ± 4 (0)	58 (43)	118 (5)
Control (none)	44 ± 4	61 ± 4	101	125
BALB/c mouse embryo RNA				
Unfractionated			51 (3)	30 (33)
Fractions eluted by				
0.5 M NaCl			46 (13)	39 (13)
0.1 M NaCl			44 (16)	25 (44)
Water			43 (18)	18 (60)
Control (none)			53	45

[a] XC plaque reduction assay was performed as described in the text. Immunofluorescence assay was made in SC-1 cells infected with the virus at MOI of 0.5, using experimental conditions described in Table 1. All RNA samples and chromatographic fractions were tested at a concentration of 10 μg/ml. Numbers in parentheses indicate percentage of inhibition relative to untreated control.

missive mouse cells with cell-free extracts (44). Genetic analysis demonstrated that the resistance transfer activity of the cell extracts segregated specifically with the corresponding *Fv-1* allele of mouse embryo cells (43). The present investigation demonstrates that RNA is the active component responsible for this transfer of *Fv-1* gene-specific resistance. The initial indication of RNA as the active component was determined by ribonuclease sensitivity. The RNA nature of the active molecule of the cell extract was supported by subsequent experiments in which extracted RNA preparations were used. When tested in SC-1 (*Fv-1*$^-$) cells, RNA extracted from NIH Swiss (*Fv-1*^{n}) mouse embryos or livers specifically inhibited B-tropic virus, but not N-tropic virus, whereas RNA extracted from BALB/c (*Fv-1*^{b}) mouse embryos or livers showed inhibition of N-tropic virus but not B-tropic virus. The reciprocity of the RNA activity has served as a specific control in these experiments. In our study a major concern has been the difficulties involved in introducing exogenous RNA molecules through the cell membrane barrier for intracellular utilization (3). Extensive experimentation to optimize bioassay conditions for reproducible and consistent data yielded three results which are particularly noteworthy.

First, SC-1 cells and mouse embryo cells in culture contain a high level of ribonuclease inhibitor activity in the cytoplasm, which is similar to the activity described by Roth (37). Labeled RNA mixed with homogenates of these cells generally showed minimal or no degradation in a brief incubation and even with added pancreatic ribonuclease at 10 ng/ml (data not given). Endogenous ribonuclease inhibitor is a relatively unstable protein, and its protective effect could be required for the stability of RNA molecules in crude cell extracts. This may explain why the resistance transfer activity of crude cell extracts was unstable in the previous studies (43, 44).

Second, our results indicate that phenol is required for the extraction of RNA, which is biologically active in our assay. The guanidine hydrochloride method of RNA isolation (5) persistently yielded inactive preparations in our hands, although these preparations all appeared undegraded by sucrose gradient sedimentation analysis. Several repeated washings of the RNA with high-salt solution are required to remove guanidine contamination, which interferes with isolation of poly(A) RNA by oligo-dT cellulose chromatography. The resistance transfer activity, however, was not recovered by such washings. Pronase digestion did not show enhancing or destroying effect on RNA isolated by either method. Phenol extraction generally does not remove the RNA-associated polyamines, nor alter the native conformation of RNA structure, and may even induce intermolecular RNA complex formation (10). These structural features of RNA isolated by phenol extraction might aid in cellular uptake and utilization.

Third, results obtained in many experiments with secondary mouse embryo cell cultures revealed that these cells were not as reliable as SC-1 cells as recipients of RNA in the bioassay. The former cell cultures degraded exogenously added labeled RNA which was derived from the same cell cultures, and the extent of uptake and degradation varied considerably according to

multiple factors such as embryo age, cell density, and medium change (M. M. Morale, J. O. Kiggans, Jr., and W. K. Yang, unpublished data). This could reflect the activity of cell membrane ribonuclease of these embryo cells. This ribonuclease activity may be different in SC-1 cells, since we were able to recover apparently undegraded labeled RNA from SC-1 cells 2 h after inoculation using a published procedure (46).

From the results presented, we believe that the *Fv-1* gene-specific resistance to murine N-tropic or B-tropic retroviruses can be transferred to permissive cells by RNA extracted from restrictive cells, and that active RNA preparations can be prepared from whole mouse embryos, cultured cells, or from livers of weanling mice. Other types of biological function have been reported to be transferred by the addition of exogenous RNA. Infection of cells by deproteinized viral RNA is well established, and several studies have focused on conditions promoting efficient uptake and utilization of viral RNAs (see review in ref. 29). Exogenously added whole cellular RNA can be detected in an undegraded state within cells (46), and reports of the use of cellular mRNA in whole-cell translation systems for interferon synthesis have been well documented (6, 14, 21, 34, 36).

We have attempted to further isolate, purify, and characterize the *Fv-1*-specific RNA. From poly(U)-Sepharose affinity chromatography it appears that the active RNA contains poly(A). This is consistent with the initial finding that most of the activity was differentially extracted into the pH 9.0 fraction by a phenol extraction method which preferentially extracts mRNA (4). However, a significant amount of activity also appeared to be present in the low-pH-extracted RNA fraction as well as in the high-salt eluate from the poly(U)-Sepharose column, especially when RNA preparations from the liver were employed. This could be due to loss of poly(A) from RNA molecules during phenol extraction (31) and the ability of the *Fv-1* gene-specific RNA to retain biological activity despite the loss of the poly(A) portion of the molecule. The sucrose gradient sedimentation analysis of the specific activity revealed a broad distribution, with a maximal activity in the 22 to 28S region. However, the determined size of the specific RNA molecule should be considered uncertain because of complicated problems related to phenol-induced RNA aggregation and possibly also because of preferential cell uptake of certain sizes of RNA. In this regard, we have attempted to quantitate RNA uptake to optimize the bioassay. It appears that a minimal concentration of 1 to 5 μg of RNA per ml is required, even with purified RNA preparations, for demonstration of the specific activity. The use of an RNA carrier may be essential for the efficient quantitative bioassay of *Fv-1* gene-specific RNA which is further purified.

Below the dose of 30 μg per 8×10^4 SC-1 cells, the amount of RNA inoculated correlated reasonably well with the extent of *Fv-1* gene-specific resistance transfer. Higher doses of RNA tended to cause nonspecific inhibition. The maximal degree of specific inhibition was 50 to 60% with most of the RNA preparations using the present assay method. Based upon an estimated RNA yield of 6 mg of RNA per g (2.5×10^8) of mouse cells, our results would indicate that this degree of specific inhibition is achieved by inoculation of approximately 15 cell RNA equivalents per SC-1 cell used for assay. Thus, it appears that an excess of RNA is added to the cells and higher concentrations lead to nonspecific effects. Incomplete inhibition of virus infection, therefore, may be due to one of several factors, including (i) competition of the *Fv-1*-specific RNA molecules with other RNA having similar characteristics of cellular uptake and utilization; (ii) competence of the cells to absorb or incorporate the added RNA under the assay conditions used; and (iii) compartmentalization where the virus (present only at MOI of approximately 0.001) enters the cell at a site distant from where the specific RNA is taken up. The latter would be expected to play a larger role if the *Fv-1* RNA acted directly rather than through translation and synthesis of a product.

The inhibitory effect of the RNA preparation on N- or B-tropic viruses was observed only within a 6- to 7-h period ranging from 2 h before to 4 to 5 h after virus inoculation, similar to the previous observation using crude cell extracts (44). This indicates that the RNA acts at an early phase of virus replication, and, if the observation represents the true *Fv-1* gene restriction mechanism, this would suggest that this host-genome restriction occurs prior to the cell-genome integration of proviral DNA, which requires a minimum of 6 h after virus infection (11, 12, 20, 40, 45). This interpretation is consistent with the observation by molecular hybridization that the integrated proviral DNA sequences are decreased in infection of *Fv-1* restrictive cells (19, 41) and also with our own DNA transfection studies, which show that the infectious activity of integrated proviral DNA is not restricted by the *Fv-1* gene of the recipient mouse cells (16, 17).

Phenotypic mixing experiments (26, 35) have shown that N- and B-tropic retroviruses contain within the virion a specific molecule which is the target for *Fv-1* restriction. Although the identity of this target molecule has not been elucidated,

in light of our understanding of the mechanism of *Fv-1* gene restriction (16, 17, 19, 41), it probably serves an important role in the formation of proviral DNA. At present, it is not clear how the RNA inhibitor demonstrated in this study may interact with such virion target molecules. However, several possible models for the mechanism of the RNA action can be considered. As a working hypothesis, the *Fv-1* gene-specific RNA could interact directly with the target molecule of the virion. Specifically, the RNA inhibitor might contain structural features resembling a specific portion of the viral RNA genome, upon which the target molecule would act during reverse transcription. Thus the *Fv-1* gene-specified cellular RNA inhibitor would compete with viral RNA for binding of the target molecule. This model predicts that large quantities of *Fv-1* gene RNA might be required for the competition and that the action of *Fv-1* gene could be abrogated in the presence of excess target molecules. It has been demonstrated (8) that *Fv-1* gene activity can be abrogated by infection of restrictive mouse cells with two infectious virus units, one of which presumably provides its genomic RNA as an mRNA for the synthesis of excess target molecules. Alternatively, it is possible that the inhibitor RNA might act indirectly by modifying the cellular function that is required for the proviral DNA formation. In this regard, it was reported that a 7S-size poly(A) RNA, isolated from chicken embryo heart and showing no mRNA activity in an in vitro protein synthesis reaction, was capable of inducing a specific change in embryonic heart differentiation (7). Therefore, the *Fv-1* RNA might act in a similar fashion as a regulatory molecule. Finally, *Fv-1* gene RNA might serve as an mRNA for the synthesis of proteins which interact with virion target molecules. We have attempted to determine whether the *Fv-1* acts through translation by testing RNA-mediated transfer in cells incubated with cycloheximide (0.5 to 2.0 μg/ml). However, virus infection was inhibited by cycloheximide even at concentrations which failed to completely stop cell protein synthesis. Studies are in progress to distinguish between the alternative modes of action.

The *Fv-1* locus is important in the control of retrovirus tumorigenesis (28) and in understanding the dominantly expressed resistance which could exist also in other species. In addition, the ability to transfer resistance may provide a new system for the study of regulation of mammalian gene expression.

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