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Somatic Cell Mutants Resistant to Retrovirus Replication: Intracellular Blocks during the Early Stages of Infection

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To identify cellular functions involved in the early phase of the retroviral life cycle, somatic cell mutants were isolated after selection for resistance to infection. Rat2 fibroblasts were treated with chemical mutagens, and individual virus-resistant clones were recovered after selection for resistance to infection. Two clones were characterized in detail. Both mutant lines were resistant to infection by both ecotropic and amphotropic murine viruses, as well as by human immunodeficiency virus type 1 pseudotypes. One clone showed a strong block to reverse transcription of the retroviral RNA, including formation of the earliest DNA products. The second clone showed normal levels of viral DNA synthesis but did not allow formation of the circular DNAs normally found in the nucleus. Cell fractionation showed that the viral preintegration complex was present in a form that could not be extracted under conditions that readily extracted the complex from wild-type cells. The results suggest that the DNA was trapped in a nonproductive state and excluded from the nucleus of the infected cell. The properties of these two mutant lines suggest that host gene products play important roles both before and after reverse transcription.

INTRODUCTION

The early phase of the retroviral life cycle consists of virus entry, reverse transcription of the viral RNA into DNA, movement into the nucleus, and insertion of the DNA into the host genome to form the integrated provirus (for a comprehensive review, see Coffin *et al.*, 1997). Although there has been considerable progress in the identification of viral receptors and coreceptors on the cell surface, the steps occurring after entry of the virus particle into the cytoplasm are still poorly understood. It remains particularly unclear what role cellular functions may play in this process. There are indications that cytoskeletal components are used by retroviruses in the early stages of entry in some cell types; for example, infection of NIH/3T3 cells by the murine leukemia viruses (MuLVs) is blocked by nocodazole, which disrupts microtubules, and by cytochalasin D, which depolymerizes the actin network (Kizhatil and Albritton, 1997). Furthermore, for most retroviruses, cell division is required to permit efficient infection. Although viral DNA can be synthesized in nondividing cells, nuclear entry and viral gene expression are blocked (Roe *et al.*, 1993). This observation suggests that the breakdown of the nuclear membrane associated with cell division, and perhaps other processes, may be required to allow access to the nucleus. Some retroviruses, notably human immunodeficiency virus type 1 (HIV-1) and other lentiviruses, are unusual in being able to infect nondividing cells, albeit at reduced efficiency as compared with dividing cells (Weinberg *et al.*, 1991; Lewis *et al.*, 1992; Lewis and Emerman, 1994). Infection by these viruses may make use of nucleoporin-mediated transport into the nucleus (Bukrinsky *et al.*, 1992).

Biochemical approaches to the study of the early phase of the life cycle are hampered by the fact that most of the virion particles that enter an infected cell are defective or at least nonproductive; the ratio of physical particles to infectious units is typically at

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least 1000:1. Thus, the vast majority of the particles in a recently infected cell may not be on a pathway to successful infection, and biochemical analysis of their properties may be misleading. Genetic approaches could provide more definitive identification of host functions needed for infection. However, only a few mutants of mammalian cells affected in early steps of infection by any virus have been identified. Some mutant cell lines initially selected for drug resistance were subsequently found to be defective in virus replication; for example, mutant cells selected for defects in endocytosis and intracellular transport exhibited increased resistance to Sindbis and vesicular stomatitis viruses (VSVs) (Robbins *et al.*, 1984). Only rarely have mutant cell lines been isolated by direct selection for resistance to virus infection (Morgan *et al.*, 1973; Taber *et al.*, 1976).

The recovery of mutant lines resistant to virus infection at useful efficiencies requires strong selections. Most somatic cell mutants have been isolated with selections for resistance to cytotoxic compounds (e.g., Gupta and Siminovitch, 1976; Funanage, 1982; Ripka and Stanley, 1986; Goldmacher *et al.*, 1987; Chopra *et al.*, 1990; Stanley *et al.*, 1990; Meuth and Harwood, 1991), and the affected gene products are restricted to a limited number of particular metabolic pathways by the availability of such compounds. More recently, however, new selectable DNA constructs have been used to permit recovery of mutant lines affected in a wider range of functions, including the responses to interferon α and γ (Pellegrini *et al.*, 1989; John *et al.*, 1991; McKendry *et al.*, 1991; Mao *et al.*, 1993). These lines were crucial to the subsequent identification of the Jak and STAT gene products in signal transduction (Velazquez *et al.*, 1992; Darnell *et al.*, 1994). Mutant lines defective in the responses to transforming growth factor β have been isolated in similar ways (Laiho *et al.*, 1990, 1991; Hocevar and Howe, 1996).

To identify cellular functions that might be important for retrovirus infection, we have devised a selection for the isolation of virus-resistant cell mutants. A permissive parental line was subjected to heavy mutagenesis by chemical mutagens and then challenged by exposure to retroviral preparations carrying a selectable marker. Rare virus-resistant clones that could not be transduced by the genetically marked retroviruses were then isolated. Two such resistant lines were examined in detail. One such line blocked challenge by retrovirus infection before reverse transcription of the viral RNA into DNA. The second line allowed normal viral DNA synthesis but retained the DNA in an abnormal state in the cytoplasm and did not allow its nuclear entry. These lines suggest that host functions play important roles in the early steps of retroviral infection, and that these functions might provide new sites for antiviral intervention.

MATERIALS AND METHODS

Tissue Culture

Rat2 cells, 293T cells, and derivative cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (HyClone, Logan, UT). NIH/3T3 cells and derivative cell lines were maintained in DMEM supplemented with 10% calf serum (HyClone).

Producer Cell Lines and Viruses

To generate ecotropic and amphotropic MuLV-thymidine kinase (TK) viruses, pMA348 (a generous gift from Drs. Manual Caruso and Arthur Bank, Columbia University), a Moloney MuLV–derived retroviral vector expressing the herpes TK gene was stably introduced into GP+E86 and GP+EnvAm12 packaging cells (Markowitz *et al.*, 1988a,b), respectively, by cotransformation with pSV2-neo DNA. Transfected cells were selected in medium containing 800 μg/ml G418. Approximately 100 G418-resistant clones from each transfection were picked individually and expanded. Supernatants from each individual clone were diluted 100-fold and used to infect naive Rat2 cells for 2 h to evaluate the titer of each producer clone. Ten days after selection in HAT (hypoxanthine, aminopterin, thymidine) medium, the Rat2 cells were stained with Giemsa, and the number of colonies was counted. One producer clone from each transfection that released the highest titer of transducing virus was chosen and expanded. The supernatants from these two clones were used as the TK virus preparations in the subsequent experiments.

Ecotropic and amphotropic MuLV-N2 viruses have been reported previously (Eglitis *et al.*, 1985; Markowitz *et al.*, 1988a,b). The pMA-Luc vector was generated by replacing the TK gene of pMA348 with the luciferase coding region recovered from p19 Luc (van Zonneveld *et al.*, 1988) by PCR. Ecotropic MuLV-Luc virus was generated by stable transformation of the GP+E86 packaging line with pMA-Luc DNA together with pSV2-neo DNA. G418-resistant clones were individually tested for virus production, and the one that produced the highest titer of virus was chosen for virus production. Ecotropic MuLV-green fluorescent protein (GFP) virus was generated by stably transfecting plasmid MFG-GFP DNA (Verhasselt *et al.*, 1998) into ecotropic phoenix producer cells (Kinsella and Nolan, 1996). Two weeks after transfection, the cells were sorted by FACS to obtain a GFP-positive population that was used as the source of MuLV-GFP virus. The virus was then used to infect GP+EnvAm12 amphotropic packaging cells to generate amphotropic MuLV-GFP producers. The method for producing VSV-G–enveloped HIV-GFP viruses has been previously reported (Naldini *et al.*, 1996). Ecotropic and amphotropic HIV-GFP viruses were produced using a similar protocol but replacing pMD.G, a plasmid expressing VSV-G, with either pSV-E-MLV-env or pSV-A-MLV-env, plasmids expressing ecotropic or amphotropic envelopes, respectively (Landau and Littman, 1992). The HIV-GFP virus with the VSV-G envelope for use in analysis of viral DNA synthesis in vivo was further purified to remove residual plasmid DNA present from the transfection. The virus preparation was first treated with 25 U/ml DNase I at 37°C for 1 h and then concentrated by centrifugation through a 25% sucrose step gradient onto a 45% sucrose cushion. The virus at the interface of the gradient was collected, diluted, and used directly for infections. To control for contaminating DNA, the undiluted virus was heat-inactivated by treatment at 80°C for 15 min and used for infection as before.

Infection of Cultured Cells and Assays for Transduction

All infections were performed in the presence of 8 μg/ml polybrene. Unless otherwise specified, infections were carried out for only 2 h. For amphotropic MuLV-GFP the infections were done for 5 h to increase the infection efficiency. Cells expressing puromycin resistance were selected by growth in medium containing puromy-

cin (5 μg/ml); those expressing neomycin resistance were selected with G418 (400 μg/ml). The level of luciferase expression was monitored by preparing lysates and assaying enzyme activity using luciferase assay reagents (Promega, Madison, WI) according to the manufacturer's instructions. Samples were read on a Lumat luminometer (Wallac, Gaithersburg, MD) to obtain light units; values were corrected to units per 20 μg of total protein. Concentrations of protein were determined using the Bio-Rad (Hercules, CA) dye reagent. Cells infected with GFP viruses were analyzed by flow cytometry to determine the number of transduced cells and the levels of GFP expression.

Random Mutagenesis and Trifluorothymidine (TFT) Selection

Rat 2 cells were first plated at very low density so that single clones could be picked. Two clones were expanded and tested for their susceptibility to both ecotropic and amphotropic MuLV-TK virus infection. Both clones were comparable with the parental cells in terms of susceptibility, cell growth rate, and morphology. One clone, Rat2-2, was used in this report.

Ethyl-Methane Sulfonate (EMS) Treatment. 10^6 Rat2-2 cells seeded on one 10-cm plate were incubated with medium containing 700 μg/ml EMS (Sigma, St. Louis, MO) at 37°C for 16 h. The treated cells were allowed to recover for 2 weeks before infection and selection.

ICR-191 Treatment. 10^6 Rat2-2 cells seeded on one 10-cm plate were incubated with medium containing 1.5 μg/ml ICR-191 (Sigma) at 37°C for 1.5 h so that 50% of the cells were killed, a dose of mutagen that results in maximum mutagenesis efficiency. The cells were allowed to recover for 4 d before being trypsinized and replated. The cells were then treated with ICR-191 three more times as described above. One week after the last treatment the cells were replated and subject to infection and selection. The mutagenized cells were repeatedly infected with ecotropic and amphotropic MuLV-TK viruses and then selected in DMEM supplemented with 10% dialyzed FBS (Life Technologies, Gaithersburg, MD) and 13 μM TFT (Sigma).

Cell Cycle Analysis

Cells (10^6) of each line were seeded in 10-cm dishes 2 d before the experiment. At 30–80% confluency, the cells were analyzed for their cell cycle profile with a cellular DNA flow cytometric analysis kit (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's instructions.

PCR Primers and Reaction Conditions

Primers to amplify GFP sequence were: forward primer, GFP-FP (5′-CTGTCAGTGGAGAGGGTGAAGGTGATGCAA-3′); and reverse primer, GFP-RP (5′-CCCAGCAGCTGTTACAAACTCAAGAAGGACCAT-3′). Primers to amplify MuLV minus strand strong stop DNA were: forward primer, MSS-FP (5′-GCGCCAGTCCTCCGATTGACTG-3′); and reverse primer, MSS-RP (5′-CGGGTAGTCAATCACTCAG-3′). Primers to specifically amplify MuLV plus strand sequence (from U5 to R) were: forward primer, PS-FP (5′-GTGATTGACTACCCGTCAGC-3′); and reverse primer, PS-RP (5′-GACCTTGATCTTAACCTGGG-3′). Primers to amplify the MuLV long terminal repeat (LTR)–LTR junction, MR5784 and MR4091, have been previously reported (Smith *et al.*, 1997). Primers to amplify the HIV minus strand strong stop DNA and the elongated plus strand DNA have also been reported (Braaten *et al.*, 1996).

PCR conditions for amplifying GFP sequence were 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. The PCR was performed with native *Taq* polymerase (Perkin Elmer, Emeryville, CA) for 25 cycles to amplify Hirt DNA, 30 cycles to amplify cell extracts and 40 cycles to amplify fractionated cytoplasmic extract. PCR conditions for amplifying MuLV minus strand strong stop DNA were 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s for 30 cycles. The same conditions were used for 25 cycles for amplifying plus strand DNA and for 30 cycles for amplifying the LTR–LTR junction. PCR conditions for amplifying HIV minus strand strong stop DNA and plus strand DNA were 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s for 25 cycles.

Fractionation of Infected Cells

Cells (4×10^7) were incubated with undiluted ecotropic MuLV-GFP virus at 4°C for 2 h and then transferred to 37°C to initiate infection. At various times after the shift, the cells were trypsinized and lysed. Two lysis methods were used. In one procedure, the cells were resuspended in a hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, and 10 μg/ml leupeptin) using 10 strokes in a Dounce homogenizer until the cell membrane was broken but nuclei remained intact, as monitored by microscopic inspection. The lysate was then centrifuged at 3300 $\times$ *g* for 15 min. The supernatant was transferred to a new tube and centrifuged again at 8000 $\times$ *g* for 20 min. This supernatant was referred to as cytoplasmic extract. The pellets from both centrifugation were pooled, and DNA was extracted for virus determination (Hirt, 1967). The other extraction method was as previously described (Brown *et al.*, 1987; Bowerman *et al.*, 1989). Briefly, 8 h after infection the cells were lysed in 0.025% digitonin in buffer A (10 mM Tris-HCl, pH 7.4, 225 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 20 μg/ml aprotinin). The lysate was centrifuged at 1000 $\times$ *g* for 3 min. The supernatant from this separation was recentrifuged at 8000 $\times$ *g* for 20 min; this supernatant is referred to as cytoplasmic extract. The pellet of the first centrifugation was resuspended in buffer A and homogenized with a ball-bearing homogenizer to break the nuclear membrane, and the preparation was centrifuged at 10,000 $\times$ *g* for 20 min. The supernatant from this separation was referred to as nuclear extract, and the pellet was referred to as secondary pellet.

To monitor the isopycnic density of the cytoplasmic extracts, samples were loaded on a linear gradient prepared with 20% sucrose in buffer (50 mM potassium phosphate, pH 7.4, 10 mM DTT, and 10 μg/ml aprotinin) and 70% sucrose in D_2O and centrifuged at 35,000 rpm (SW55 rotor; Beckman Instruments, Palo Alto, CA) for 20 h. The gradient was divided into 10 fractions with 500 μl in each fraction.

RESULTS

Selection for Virus-resistant Mutant Cell Lines

A strategy was devised for the isolation of somatic cell mutants resistant to infection by retroviruses (Figure 1). Cells susceptible to virus and deficient in TK were mutagenized and then infected with a retroviral vector carrying the herpes virus TK gene. The parental cells would be infected and become TK positive, whereas any rare mutant virus-resistant cells would remain TK negative. The mutant cells could then be recovered by growth in TFT, which would kill the infected TK-positive cells but spares the uninfected TK-negative mutants.

The Rat2 cell line was chosen as parent for the mutagenesis for several reasons: the line is highly susceptible to the MuLVs and expresses both the ecotropic and amphotropic virus receptors; it is relatively stable genetically and has a nearly diploid karyotype (N = 44); and it is deficient for TK, a powerful selectable and counterselectable marker. The cells were mutagenized by exposure to high doses of a chemical

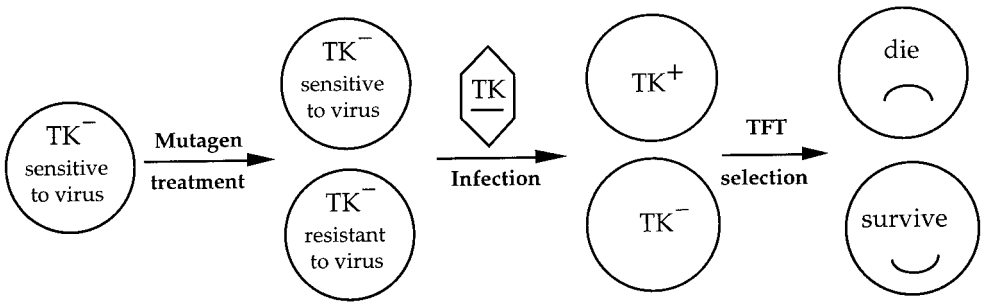

Figure 1. Schematic representation of the strategy for isolating mutant cells resistant to retroviral infection. TK−, thymidine kinase deficient; TK+, thymidine kinase proficient.

mutagen and allowed to recover and express the phenotype for several days. The cultures were then challenged with a mixture of two virus preparations, containing either the ecotropic or the amphotropic envelope protein, and delivering the herpes virus TK gene via the same retroviral vector. The use of a mixture of viruses was designed to infect those uninteresting mutant cells that lacked either one of the two receptors. Repeated infection was found helpful to ensure delivery of the TK gene to the bulk of the cells. Finally, the rare TK-negative cells were selected by plating in medium containing TFT. The background of surviving cells in the selection protocol was as low as 1 in 10^5 cells plated in this medium.

Isolation of Virus-resistant Clones

Initial efforts to isolate virus-resistant mutants used the mutagen EMS, which mainly induces substitutions, both transversions and transitions. After infection with the TK virus preparations, ~10^7 cells were plated in TFT medium and gave rise to ~50 surviving colonies. These clones were individually expanded into large cultures and retested for virus resistance by challenge with a viral vector preparation transducing the neomycin resistance marker N2 in an ecotropic virus coat (Eglitis *et al.*, 1985). A small number of the clones exhibited significant resistance, showing 10- to 300-fold reductions in the efficiency of transduction to G418 resistance (Table 1). Control experiments without mutagen did not produce any such resistant colonies, suggesting that the EMS did increase the mutation frequency. The phenotype of these clones, however, was not stable. As the cells were cultured over the subsequent months, the resistance gradually disappeared. Similar observations have been made with other mutants isolated by EMS mutagenesis (Nakamura and Okada, 1983).

Similar experiments were performed using the chemical mutagen ICR-191, an acridine half-mustard that induces frameshift mutations and chromosomal rearrangements (MacInnes *et al.*, 1982) and was used to isolate stable mutants affected in the Jak-STAT pathways (Pellegrini *et al.*, 1989; John *et al.*, 1991; McKendry *et al.*, 1991; Mao *et al.*, 1993). Approximately 2×10^7 cells, divided into 20 separate pools, were subjected to multiple rounds of exposure to ICR-191. The dosage of mutagen was controlled to induce killing of ~50% of the cells at each round. After a total of four rounds of mutagenesis the cells were allowed to recover for 2 weeks, and then 2×10^6 cells in each pool were repeatedly infected with the ecotropic and amphotropic viral vectors over the course of 2 d. Finally, the cells were grown in medium containing 13 μM TFT to kill the infected TK-positive cells and select for

Table 1. Selection of virus-resistant cell clones

Mutagen	No. of cells subjected to selection	No. of surviving colonies[a]	No. of resistant clones[b]	No. of stable resistant clones[c]
No mutagen	2×10^7	63	0	
EMS				
Exp. 1	1×10^7	72	8	0
Exp. 2	8×10^6	29	3	0
ICR-191	2×10^7	75	9	2

[a] Total number of colonies surviving TFT selection.

[b] The surviving clones were individually tested for resistance to neo^R viral vector. Those demonstrating >10-fold reduction in virus sensitivity were considered resistant clones.

[c] The resistant clones were cultured for >8 weeks and retested for resistance to neo^R viral vector. Those retaining resistance were considered stable clones.

Table 2. Growth properties of virus-resistant cells

Cell line	Td (h)[a]	% of cells[b]		
		G0/G1	S	G2/M
Rat2	16.8			
RC-2	21.5	63	23	14
R3-2	29.5	66	19	15
R4-7	20.0	43	30	27

[a] Td, doubling time.

[b] Asynchronously dividing cells were stained with propidium iodide and analyzed by flow cytometry.

any virus-resistant mutants. More of the viral vectors were added during the selection to target any susceptible cells and lower the background of survivors. Approximately 100 clones were isolated and retested for resistance to infection by ecotropic N2 virus. A total of nine clones exhibited >10-fold resistance to infection; two of these, clones R3-2 and R4-7, showed the highest level of resistance and stability and were chosen for further characterization.

Growth Properties of Mutant and Control Clones

After mutagenesis, the unselected cell cultures were observed to grow somewhat more slowly than the parental population. Virus replication is known to depend on cell division, and thus a simple reduction in the growth rate or an alteration in the cell cycle behavior could cause virus resistance. To examine this possibility, the doubling times of clones R3-2 and R4-7 were determined and compared with two clones randomly picked from the mutagenized cultures without infection or TFT selection. These clones, RC-1 and RC-2, were fully sensitive to virus infection (see below). All the clones, both the virus-resistant and the virus-sensitive lines, showed modest reductions in their growth rate compared with the parental population (Table 2). Thus, the reduction in growth rate by itself did not cause virus resistance, suggesting that R3-2 and R4-7 exhibited an additional or unrelated mechanism of resistance.

To characterize the growth behavior of the clones further, the cells were stained with propidium iodide and examined for DNA content by flow cytometry. The distribution of cells in each stage of the cell cycle was determined (Table 2). For R3-2 cells, the distribution in each phase was very similar to controls, whereas for R4-7 cells, more cells were found in the S and G2/M phases than controls. The altered pattern did not correlate with the doubling times of the clones or with the virus resistance of the mutants. Thus, there was no simple alteration in the growth of these clones that could on its own account for their resistance.

Table 3. Resistance of mutant cell lines to neo^R viral vectors

Cell line	Exp. 1[a]		Exp. 2[a]			
			Eco-N2		Amp-N2	
	Eco-N2	Amp-N2	Undiluted	1:10	Undiluted	1:10
RC-1	82	46				
RC-2	119	70	TMTC[b]	>2000	TMTC[b]	884
R3-2	0	1	58	3	2	0
R4-7	1	0	122	26	93	7

[a] Number of drug-resistant colonies after infection with the indicated viral preparation.

[b] TMTC, too many to count.

Measurement of the Level of Resistance to Virus Infection

To determine the magnitude of the resistance to virus infection in a single round, cultures were challenged with preparations of a viral vector expressing the neo^R gene (the N2 vector; Eglitis *et al.*, 1985) in either ecotropic or amphotropic envelopes. The infected cells were plated in medium containing G418, and the number of transduced colonies was counted after 10–14 d. Both R3-2 and R4-7 were highly resistant to both ecotropic and amphotropic virus infection (Table 3). Mutant R3-2 was ~1000-fold resistant, and mutant R4-7 was ~100-fold resistant, compared with the RC-1 and RC-2 controls. Similar results were obtained with a puromycin-transducing virus (our unpublished results).

Bypass of the Virus Resistance by DNA Transformation

The resistance to virus manifest by clones R3-2 and R4-7 could in principle be due to blocks early in infection, before establishment of the integrated provirus, or later in infection, during expression of the provirus. To distinguish between these possibilities, the ability of the cells to express viral constructs was assessed after calcium phosphate–mediated transformation by cloned proviral DNAs. Clones R3-2 and R4-7 were readily transformed by the proviral DNA of the N2 vector, yielding slightly more drug-resistant colonies than the controls (Table 4). The variation exhibited by the clones was within the range usually attributable to variation in transfection efficiency. Similarly, the clones were able to express puromycin resistance at normal levels from the proviral DNA construct (Table 4). Thus, there was no apparent defect in transcription from the provirus or other steps involved in expression of the transduced markers. The block to virus-mediated transduction in both mutant

Table 4. Functional expression of drug resistance after DNA-mediated transformation of mutant cell lines

	N2[a]			pBabe-puro[b]	
	Exp. 1		Exp. 2		
Cell line	10 μg	1 μg	1 μg	10 μg	1 μg
RC-2	170	31	5	187	17
R3-2	89	38	50	NA[c]	45
R4-7	250	91	15	500	79

[a] Number of colonies growing in G418-containing medium after transformation by the indicated amount of DNA.

[b] Number of colonies growing in puromycin-containing medium after transformation by the indicated amount of DNA.

[c] NA, not available.

lines was thus probably manifest during the early phase of infection.

Similar experiments were also performed with a virus vector expressing the luciferase gene. The cells were infected and grown for 2 d, and lysates were prepared and assayed for luciferase activity as a measure of infection efficiency. In these experiments the R3-2 and R4-7 clones both exhibited only a 20-fold reduction in luciferase expression compared with controls (Table 5). Thus, in this short-term assay the mutant clones exhibited substantial resistance to infection, although less than in the long-term assay for drug-resistant colony formation that required stable expression of the provirus. Introducing the same construct by DNA-mediated transformation, as with the selectable viruses, showed no block to expression of the reporter gene (Table 5).

Resistance to HIV Pseudotypes using Any of Three Envelope Proteins

To explore the breadth of the resistance, the mutant clones were challenged by infection with HIV-based retroviral vectors using various envelope proteins. An HIV vector expressing the GFP was used to transfect 293T cells along with one helper plasmid expressing HIV Gag and Pol genes, and another expressing either the MuLV ecotropic envelope, the MuLV amphotropic envelope, or the VSV-G envelope protein. MuLV vectors expressing GFP in MuLV particles were used for direct comparison. These various virus preparations were used to infect clones R3-2 and R4-7, and the number of infected cells was determined by flow cytometry. As expected, both clones were highly resistant to the MuLV vectors, yielding ~30-fold fewer infected cells than the control line (Figure 2). The R3-2 clone was somewhat more resistant to the MuLV viruses than R4-7. Both clones were also highly resistant to the HIV vectors, using any of the three envelopes (Figure 2). Thus, the clones showed resistance to two very different retroviruses. Curiously, with the HIV-1 vectors, the R4-7 clone was consistently more resistant than the R3-2, just the reverse of the behavior with MuLV vectors. This result indicates that the magnitude of the resistance showed a dependence on the origin of either the transducing viral genome or the viral core proteins. The fact that the cells were resistant to viruses using any of three receptors again suggests that the block is not likely to be at the level of the receptor.

Analysis of Proviral DNA Synthesis in Mutant Cells

To identify the position of the blocks to infection, the course of viral DNA synthesis was examined after acute infection of mutant and control cells. Cells were infected with ecotropic MuLV-GFP vector at various dilutions. At different times after infection, cells were lysed, low-molecular-weight DNA was extracted, and the viral DNA was analyzed by PCR followed by gel electrophoresis. In the RC-2 control cells, viral DNA was easily detected using primers specific for the GFP sequences, and the levels of the amplified DNA product correlated well with the levels of input virus, demonstrating that the assay was not saturated but

Table 5. Expression of luciferase activity after infection or transduction with MuLV-Luc viral vector

	Virus infection[a]			DNA transfection[b]		
Cell line	Prep 1	Prep 2	Prep 3	20 ng	200 ng	2 μg
RC-2	11,043	12,698	5,188	1,609	11,586	111,296
R3-2	400	522	191	7,410	57,421	498,101
R4-7	428	323	146	4,135	19,626	178,160

[a] Cells were infected with different preparations of ecotropic MuLV-Luc virus, and lysates were assayed for luciferase activity. The values are normalized to input protein.

[b] Cells were transfected with the indicated amounts of MuLV-Luc DNA, and lysates were assayed for luciferase activity. The values are normalized to input protein.

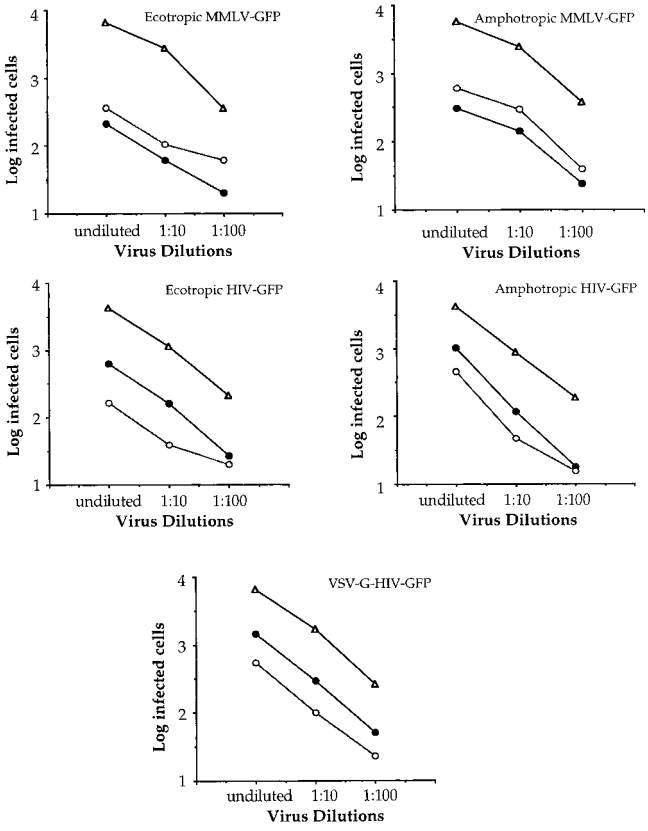

Figure 2. Resistance of mutant cells to GFP virus infection. RC-2 cells (open triangles), R3-2 cells (closed circles), and R4-7 cells (open circles) were infected with the indicated viruses at indicated dilution. The number of infected cells, as indicated by expression of GFP, was determined by flow cytometry.

responded to input DNA (Figure 3A). Comparable levels of viral DNA were detected in R3-2 cells, suggesting that this mutant clone had no defect in synthesis, at least of the minus strand of the genome. To examine later steps in reverse transcription, PCR primers that specifically amplify plus strand DNA products were used (Figure 3B). Plus strand DNA was formed at normal levels in the R3-2 cells, indicating that most of the course of reverse transcription occurred normally and that later steps in infection must be affected.

In contrast to the results with R3-2, only very low levels of MuLV viral DNA were detected in R4-7 cells using the GFP primers, suggesting a block before formation of long minus strand DNAs (Figure 3A). The amount of DNA was reduced ~30-fold from that seen in the control line, consistent with the reduction in GFP-positive cells after infection. To examine earlier steps in reverse transcription, primers were used that detect minus strand strong stop DNA, the first detectable product of DNA synthesis. The levels of this DNA after infection with ecotropic MuLV-N2 or MuLV-GFP vectors were also dramatically reduced, ~30- to 100-fold, compared with the control cells (Figure 3C). Thus, the block to infection in R4-7 cells was at or before the earliest step of reverse transcription. This result places the defect close to virus entry into the cell. To repeat the test with a different receptor, R4-7 and control cells were infected with amphotropic MuLV-GFP, and the viral DNA was again examined using PCR primers specific for GFP. The viral DNA was dramatically reduced in the mutant (Figure 3D), to a similar extent as with the ecotropic virus. To further rule out potential defects in the receptor, R4-7 cells were transformed with a DNA construct overexpressing the ecotropic receptor and then challenged with ecotropic vectors; there was no change in the resistance (our unpublished results). These findings strongly suggest that resistance in the R4-7 cells is not at the level of the receptor but is soon after entry and before reverse transcription.

The early steps in infection by HIV-1 are broadly similar to those for MuLV, but the nature of the preintegration complex may differ, and there are significant differences in that HIV-1 can infect nondividing cells. To test whether HIV-1 infection was blocked at similar stages as MuLV infection, VSV-G–pseudotyped HIV-GFP virus was used to infect the mutant cells, and the course of viral DNA synthesis was analyzed by PCR (Figure 3E). As seen with MuLV-GFP infection, formation of the HIV-1 DNA was dramatically inhibited in the R4-7 clone, suggesting a similar block soon after entry and before reverse transcription. Both minus strand strong stop DNA and elongated plus strand DNAs were blocked. As with MuLV-GFP, synthesis of the HIV-1 DNA was normal in the R3-2 cells. Both strong stop DNA and elongated DNAs were formed normally, suggesting a block only after completion of reverse transcription.

Formation of Circular Viral DNAs

After completion of reverse transcription, linear viral DNA enters the nucleus and gives rise to circular DNA forms containing one or two copies of the LTR. These circular DNAs are not intermediates on the normal pathway to formation of the integrated provirus (Ellis and Bernstein, 1989; Lobel *et al.*, 1989) but nevertheless serve as hallmarks of the entry of the DNA into the nucleus. To test for formation of circular MuLV DNAs, the levels of the two-LTR circle in acutely infected cells were estimated using PCR primers located in the U5 and U3 portions of the LTR. In control RC-2 cells, circular DNA was first detected as soon as 12 h after infection, and higher levels were found at later times (Figure 3F). In mutant R3-2 cells, virtually no circular DNA was detectable even 36 h after infection, suggesting a major block after reverse transcription and before nuclear entry.

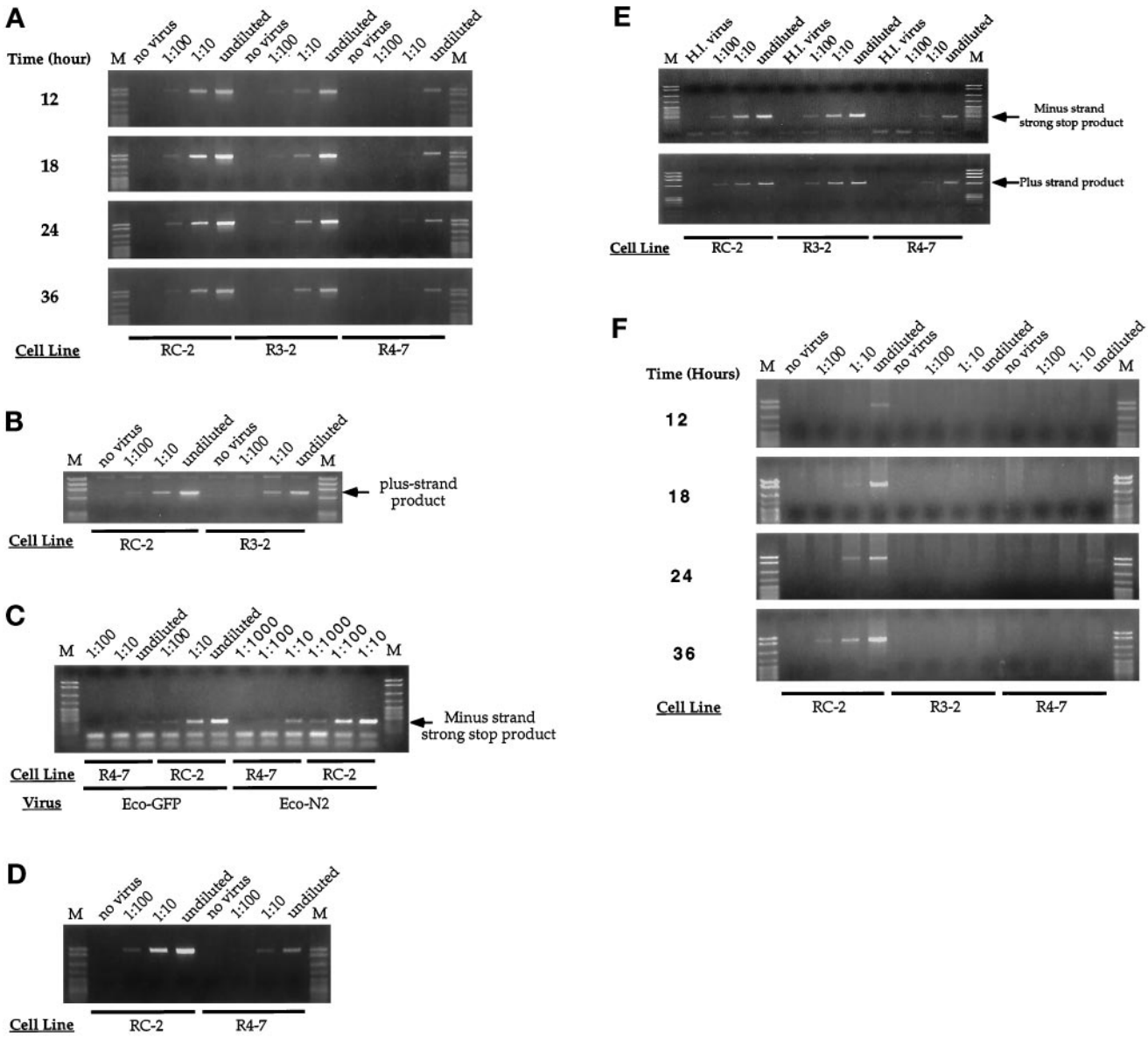

Figure 3. Analysis of viral DNA in infected mutant cells. RC-2 and mutant cells were infected with various dilutions of virus, and the amount of viral DNA synthesized in the infected cells was measured by PCR under conditions controlled such that the yield of PCR product was proportional to the input DNA. (A) Cells were infected with MuLV-GFP virus at various dilutions. At different time points the viral DNA in the infected cells was extracted and detected by PCR using primers that specifically amplify the GFP sequence. (B) The same preparations at the 24-h postinfection time point in A were used as templates to detect the synthesis of plus strand viral DNA in the infected cells. The migration position of the PCR product is indicated. (C) Cells were infected with either ecotropic MuLV-GFP or ecotropic MuLV-N2 virus at various dilutions, as indicated. Twenty four hours after infection the cells were collected, and minus strand strong stop DNA was detected by PCR. The migration position of the PCR product is indicated. (D) RC-2 and R4-7 cells were infected with amphotropic MuLV-GFP virus at various dilutions. Twenty-four hours after infection, viral DNA synthesis was analyzed by PCR using primers that amplify GFP sequences. (E) Cells were infected with VSV-G–pseudotyped HIV-GFP virus at various dilutions, as indicated. At 24 h after infection, cells were collected, and minus strand strong stop DNA and plus strand DNA were detected by PCR. The migration positions of the PCR products are indicated. H.I. virus, heat-inactivated virus was used as a control for plasmid DNA contamination of the virus. (F) The same preparations from A were analyzed by PCR using primers that specifically amplify the LTR–LTR junction to detect circular DNAs in the nucleus.

Although R4-7 cells synthesized very little viral DNA, similar tests were performed to test for circular forms. The R4-7 cells did produce low, but detectable, levels of this DNA (Figure 3F). The small amount of circular DNA was consistent with the reduced amount of initial products of reverse transcription. Thus, these

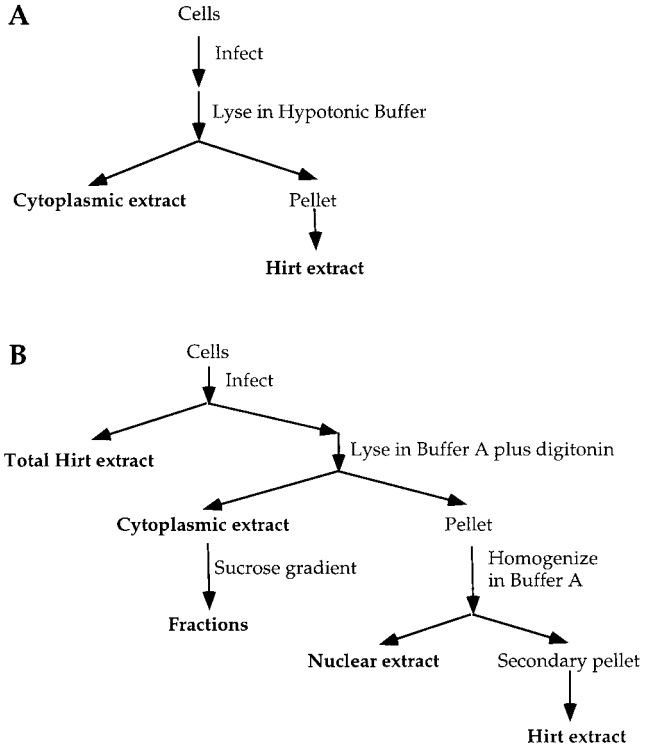

Figure 4. Schematic representation of the procedure for fractionating infected cells. RC-2 and R3-2 cells were infected with undiluted ecotropic MuLV-GFP virus and extracted with either hypotonic buffer (A) or buffer containing digitonin (B).

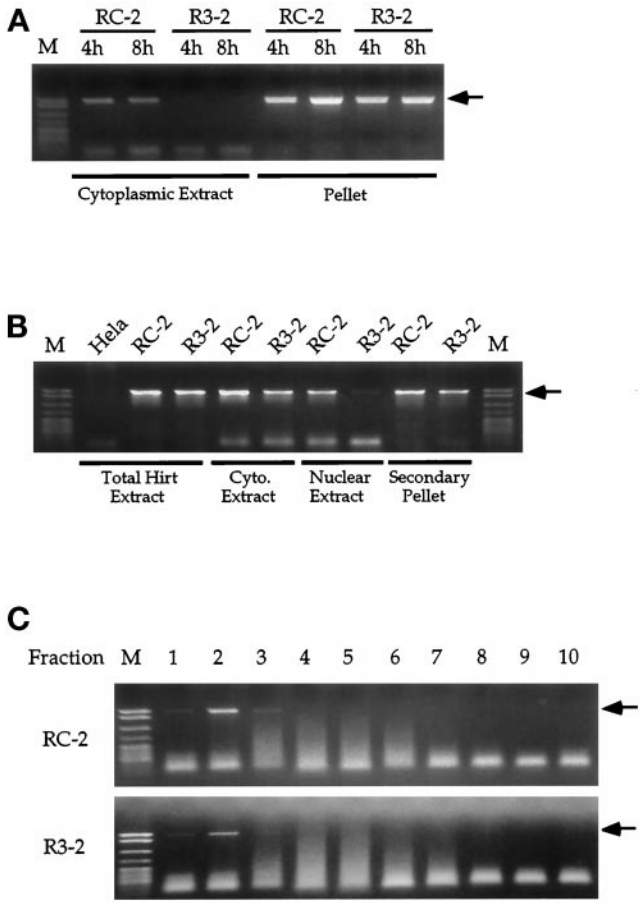

Figure 5. Analysis of viral DNA in the fractionated cell extracts by PCR using primers that amplify GFP sequences. The migration positions of the PCR product are indicated with arrows. (A) Four or 8 h after infection cells were collected and fractionated. The low-molecular-weight DNA in the pellet (Hirt extract) was resuspended in 20 μl of Tris-EDTA plus 10 μg/ml RNase A. Five of 300 μl of cytoplasmic extract or 1 of 20 μl of the pellet DNA were PCR amplified to detect viral DNA. (B) Eight hours after infection the cells were collected by trypsinization. One-third of the cells were used to extract total DNA by the Hirt procedure. The rest of the cells were fractionated as described in Figure 4B. Five of 300 μl of each extract or 1 of 60 μl of the total DNA were used to assay viral DNA by PCR. (C) The cytoplasmic extracts from B were fractionated by centrifugation on a 20–70% sucrose gradient. The gradient was equally divided into 10 fractions of 500 μl each. Five microliters of solution from each fraction were used to detect viral DNA by PCR.

cells apparently translocated into the nucleus a normal proportion of the small amount of DNA that was synthesized.

Characterization of the Viral DNA in R3-2 Cells

The failure of the R3-2 cells to form circular viral DNAs suggested that nuclear import or intracellular transport of the DNA might be affected. To determine the state of the unintegrated viral DNA, cell extracts were prepared in various conditions, fractionated, and assayed for the presence of the DNA. Cells were exposed to ecotropic MuLV-GFP virus for 2 h at 4°C and then transferred to 37°C to allow infection to proceed synchronously. At 4 or 8 h after infection, cells were harvested and lysed with a mild hypotonic buffer, and the lysates were fractionated by centrifugation into a soluble fraction and an insoluble pellet (Figure 4A). In the control RC-2 cells, approximately equal amounts of viral DNA were recovered in the soluble and insoluble fractions (Figure 5A). In contrast, in the R3-2 cells, although normal levels were recovered in the insoluble fraction, almost no DNA was detected in the soluble fraction. This result suggests that in R3-2 cells the viral preintegration complex was trapped in a state that could not be solubilized by hypotonic buffer.

Cell lysates were also prepared using harsher conditions in buffer containing digitonin (Figure 4B). These lysates were fractionated by low-speed centrifugation into a soluble extract and a pellet containing nuclei, membrane, cytoskeleton, and associated proteins. Under these conditions, viral DNA was detected in the soluble fraction of both the RC-2 and R3-2 cells, and similar levels were also detected in the pellet (Figure 5B). The pellet was resuspended in the same buffer, extracted with a Dounce homogenizer, and

separated by centrifugation again into a soluble nuclear extract and a secondary pellet. Although viral DNA was readily detected in the nuclear extract of the control RC-2 cells, very little was recovered in this fraction in the R3-2 cells (Figure 5B). This result is consistent with a failure of the DNA to move into the nucleus. No difference in the amounts of viral DNA in the secondary insoluble pellet was detected between the two cell lines.

To test for any profound differences in the preintegration complex extracted in the harsher conditions, the digitonin-soluble fractions from both cell lines were analyzed by equilibrium density gradient centrifugation (Figure 5C). The DNAs from both lines localized at similar positions in the gradients, at a density of ~1.32 g/ml, indicating no gross difference in the complexes.

DISCUSSION

The procedure devised for the isolation of virus-resistant mutants combined an efficient transduction of cells by viruses carrying the TK gene with a powerful selection using the cytotoxic thymidine analogue TFT. The transducing virus preparations were applied repeatedly to the cells to maximize the number of parental cells that were infected. A mixture of two viruses targeted to different cell surface receptors, ecotropic and amphotropic viruses, was used to prevent the recovery of uninteresting mutants that lacked one or the other of the two receptors. The TFT itself was a highly efficient agent for the elimination of the transduced cells. The combined selection resulted in the survival of ~1 in 10^5 of the input cells, a low enough background that the true virus-resistant cells could be identified among the survivors by direct test after cloning. The two mutagens used, EMS and ICR-191, generated approximately equal numbers of virus-resistant cells—a few such mutants out of ~100 surviving clones—but with very different properties. The phenotypes of the EMS-induced clones were quite unstable, and during passage the cells reverted to normal virus sensitivity. The ICR-191–induced clones, however, were relatively stable, and even after prolonged passage the lines continued to show high level resistance.

The two cell lines characterized in detail, R3-2 and R4-7, showed very significant blocks to virus infection. When tested with either ecotropic or amphotropic viruses expressing the neomycin resistance gene, the cells showed ~1000- and 100-fold lower titers, respectively, than control cells. Somewhat lower resistance was observed with viruses transducing other markers, the GFP or luciferase genes, as scored by flow cytometry or enzyme assay. The basis for this difference is unclear but may reflect the difference in duration and level of expression required for the two types of markers. Transduction of the neo^R marker requires long-term expression sufficient to mediate cell growth over a 2-week period, whereas GFP or luciferase transduction only requires short-term expression. In any event, the level of resistance manifest by these lines suggests that it may be possible to complement or suppress the defect with DNAs and thus to select from a large library of cDNA expression constructs for those clones that restore normal susceptibility to infection. The level of resistance, however, is probably not as strong as would be required to allow the one-step selection of one such clone from a complete library of perhaps 10^6 cDNAs, so repeated cycles of selection may be required.

Virus infection of both mutant clones examined here was blocked during the early phase of the life cycle. The blocks were observed no matter whether ecotropic or amphotropic envelope or even the VSV-G protein was used for entry, suggesting a postentry step that is independent of any particular receptor. Remarkably, the cells were also resistant to infection by HIV-1 vectors, suggesting an alteration in a very general and basic aspect of retrovirus entry. The courses of infection by MuLV and HIV-1 are different in many regards: ecotropic MuLV entry is inhibited by compounds that block endosomal acidification, suggesting intracellular fusion of viral and host membranes (Marsh and Helenius, 1989; Nussbaum *et al.*, 1993), whereas HIV-1 fusion occurs at the cell surface; and MuLV infection requires cell division (Roe *et al.*, 1993), whereas HIV-1 does not (Lewis and Emerman, 1994). The fact that the mutant cells were resistant to both viruses indicates that a common, shared aspect of infection was blocked. It is also noteworthy that the two clones showed different levels of resistance to HIV-1 and MuLV; R3-2 was more resistant to MuLV, whereas R4-7 was more resistant to HIV-1. Thus, there was some virus specificity in the altered host functions.

The R4-7 cell line prevented an early step of virus infection, before reverse transcription could form the preintegrative double-stranded DNA. Analysis of the DNA suggested that even the earliest intermediate, the minus strand strong stop DNA, was not synthesized. This stage of the life cycle, sometimes termed "uncoating," is poorly understood, but many mutant viruses are also blocked at this time. Mutations in the MA and CA portions of the viral Gag gene (Crawford and Goff, 1984; Hsu *et al.*, 1985; Yu *et al.*, 1992; Craven *et al.*, 1995; Alin and Goff, 1996; Casella *et al.*, 1997; Kiernan *et al.*, 1998), mutations that block processing of the Gag precursor to the mature forms (Crawford and Goff, 1985; Katoh *et al.*, 1985), and mutations affecting the polymerase activity of reverse transcriptase (RT) itself (Schwartzberg *et al.*, 1984) are all arrested at this stage. In the case of HIV-1, mutations that block the interaction of Gag with cyclophilin A similarly

block infection at this time (Braaten *et al.*, 1996). The corresponding behavior of the R4-7 line suggests that some host protein that interacts with Gag or RT may be required for normal DNA synthesis. A plausible function for such a protein would be the opening of the intracellular virion core to allow triphosphates to enter and for reverse transcription to begin, or the direct activation of RT. This protein would have to interact with both MuLV and HIV-1 proteins, because both are blocked at a similar stage, before DNA synthesis. There seemed to be no additional blocks after MuLV DNA synthesis, because the reduced amount of linear viral DNA formed in the infected cells gave rise to correspondingly and only the appropriately reduced levels of circular DNA forms.

The R3-2 cell line allowed normal reverse transcription of both MuLV and HIV-1 DNAs, and infection was blocked at later stages. The resulting preintegrative MuLV DNA showed very aberrant properties. Whereas a substantial fraction of the DNA in wild-type cells was extracted in soluble form without detergent, the bulk of the DNA in R3-2 cells was trapped in insoluble fractions. This DNA was readily extracted with nonionic or ionic detergents. Furthermore, there was no formation of the two-LTR circular DNA, a hallmark of entry into the nucleus, and no DNA could be extracted from an initial crude nuclear fraction. These results suggest that the trapped viral DNA was unable to properly move into the nucleus in the mutant cell line. It is possible that the DNA is bound to cytoskeletal components in an inappropriately tight association. There is considerable evidence that the incoming virus complex may be carried into the cell by cytoskeleton-associated transport mechanisms (Kizhatil and Albritton, 1997), and this process may be abnormal in the mutant line. The fact that there was a quantitatively different effect on MuLV and HIV-1 is consistent with the possibility that the Gag or Pol proteins of the incoming virion might be the target.

The effect of the mutation in the R3-2 cells is reminiscent of the effects of another host gene, the Fv1 locus, which causes a dominant resistance to virus infection (for reviews, see Jolicoeur, 1979; Goff, 1996). Cells expressing the $Fv1^b$ allele are resistant to N-tropic viruses, and those expressing the $Fv1^n$ allele are resistant to B-tropic viruses. The determinants of viral tropism lie in the CA region of the Gag gene (DesGroseillers and Jolicoeur, 1983; Ou *et al.*, 1983). Interestingly, the course of infection is blocked by Fv1 soon after viral DNA synthesis, and before DNA integration (Jolicoeur and Baltimore, 1976); furthermore, the preintegrative DNA is not extracted efficiently from nonpermissive cells using standard buffer conditions (Pryciak and Varmus, 1992). Thus, the R3-2 resistance function seems to trap the viral DNA in a stage of infection and in a state very similar to those of Fv1 resistance. There is no other evidence, however, to suggest that the R3-2 block is directly related to Fv1. Recently the Fv1 locus has been cloned and shown to encode either of two variants of an endogenous retroviral Gag protein (Best *et al.*, 1996); the mechanism of its action, however, remains unclear.

Preliminary efforts to determine whether the resistance mutations in R4-7 and R3-2 are genetically dominant or recessive to the wild-type alleles by analysis of somatic cell hybrids have been inconclusive. The mutant cells were fused with normal human cells, and the heterokaryons were challenged by infection with ecotropic viral vectors. The mutant rat cells were resistant by virtue of their intracellular block, and the human cells were resistant because they lack the ecotropic virus receptor; if the mutations in the rat cells were recessive, the heterokaryons would be virus sensitive. Control experiments, however, suggested that the fused cells were not reliably healthy enough to always support good infection, and other approaches will be required to address this issue more definitively. Recent efforts to relieve the blocks by transformation with cDNA constructs have resulted in the recovery of specific antisense cDNAs that restore virus sensitivity. These results suggest that the lines may contain a dominant-acting resistance gene, although other models can explain these observations. The further analysis of the block in these cells, and the identification of the genes responsible, should provide important information about the course of virus infection.

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