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Isolation and Properties of Moloney Murine Leukemia Virus Mutants: Use of a Rapid Assay for Release of Virion Reverse Transcriptase

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A rapid assay for retroviral reverse transcriptase activity released into the culture medium by infected cells was developed. With the assay, 4,000 clonally infected cell lines could be tested in a few hours. We have adapted the assay for use as a screen for the detection of spontaneous viral mutants. Mutants of Moloney murine leukemia virus have been isolated which (i) produce a thermolabile reverse transcriptase, (ii) are temperature sensitive for release of enzyme activity, or (iii) can only productively infect cells already producing *gag*-related polypeptides. The assay has also been useful for the isolation of nonproducer cells infected with various replication-defective transforming viruses.

Most knowledge about retroviruses has been gained by direct biochemical analyses of virion nucleic acids and proteins. Very little is known about the function of each virus-encoded protein in the life cycle, largely because very few mutants mapped to specific proteins and affecting specific functions have been isolated. In the murine system, there have been temperature-sensitive mutants isolated with impaired *gag* and *gag-pol* cleavage and reduced virus release at 39°C (16, 19). Nonconditional mutants with altered protein phenotypes have been described (15). There also have been reports of early mutants found to have temperature-sensitive polymerase and RNase H function (17), and a nonconditional *pol* mutant has been described recently (4). Finally, mutants which release particles which cause no plaque formation in the XC assay (XC- mutants) have been described (5, 9, 10). Few of these mutants have been mapped on the genome or ascribed to proteins, and the lesion in most of the early and late mutants remains unknown. More mutants have been described and mapped in the avian system. *gag* processing mutants have been described, and nonconditional mutants in *gag* support the idea that *gag* proteins are required for successful virion assembly (8). The behavior of a mutant in p15 confirms its probable role as the protease responsible for *gag* and *gag-pol* cleavage (11). *env* mutants have been described (7, 14) which confirm the following about the *env* glycoprotein: its role in determining subgroup specificity, its necessary presence for viral infectivity, and its unimportance in virion assembly. Lastly, polymerase mutants with thermolabile activity are numerous (1, 6), and mutants in *pol* maturation have been discussed (2).

We have begun constructing a library of mutants of Moloney murine leukemia virus (M-MuLV) blocked in various stages of the life cycle, which may be useful in assigning functions to the known proteins. To detect mutants, a rapid assay for the virus-encoded reverse transcriptase was developed by which several thousand clones of infected cells could be assayed in an afternoon.

MATERIALS AND METHODS

Cells and viruses. The NIH/3T3 cell line was grown in Dulbecco's modified Eagle medium containing 10% calf serum. M23 cells were derived in this laboratory (15). These cells contain a defective provirus of M-MuLV which encodes only the $Pr65^{gag}$ protein. M-MuLV clone 1 was the source of infectious virus preparations and was used as our wild-type virus (3). Rauscher MuLV *ts*29 (R-*ts*29) was a gift from S. Aaronson (17). Virus was titrated by the XC plaque assay (13). Samples to be assayed at temperatures other than 37°C were first preincubated at the indicated temperature for 45 min in medium containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.35, and then applied to the cells at the same temperature.

Rapid reverse transcriptase assay. Clones were prepared by infection of NIH/3T3 cells at a multiplicity of approximately 0.3 PFU/cell for 2 h at 37°C. The cells were then trypsinized, counted, and seeded into 96-well cloning trays at 0.3 cells per well (in a volume

of 0.2 ml per well). The clones were allowed to expand for 10 to 12 days. A replicating device (Flow Laboratories, Inc.) was used to remove 10 μl of the supernatant medium from each well and add it to 50 μl of a reaction cocktail aliquoted into disposable 96-well trays (Dynatech Laboratories). The cocktail gave the following final concentrations: 50 mM Tris-hydrochloride, pH 8.3; 20 mM dithiothreitol; 0.6 mM $MnCl_2$; 60 mM NaCl; 0.05% Nonidet P-40; 5 μg of oligodeoxythymidylic acid per ml; 10 μg of polyriboadenylic acid per ml; and 10 μM α[^{32}P]dTTP (specific activity, 1 Ci/mmole). The reaction was incubated at the appropriate temperature for 1 to 2 h, and 10 μl of the mixture was spotted directly onto sheets of dry DEAE paper (DE-81; Whatman, Inc.) in a 96-spot grid, using the mechanical replicator. The paper was then washed with gentle rocking at room temperature three times in 500 ml of 2× 0.3 M NaCl–0.03 M sodium citrate for 15 min each and then twice in 500 ml of 95% ethanol. The paper was dried and exposed to X-ray film (XR-5) at −70°C with an intensifying screen (Dupont Lightning Plus).

Infection of replicate trays of cells. Virus in the culture medium above clones of cells was passed onto recipient cell lawns as follows. The recipient 96-well trays were seeded with 0.1 ml of a cell suspension (10^4 cells/ml) in medium containing 2 μg of Polybrene per ml. After 1 day, 10 μl of the medium in each cloning well of a tray of donor cells was transferred to the recipient trays, using the replicating device; the infected cells were incubated for 1 day, and then the medium was changed to normal medium (lacking Polybrene). After 1 more day, the recipient cells were assayed for reverse transcriptase by the rapid procedure.

Conventional reverse transcriptase assays. The conventional reverse transcriptase assay (12) was carried out by incubating 50 μl of the culture medium to be assayed with 100 μl of a cocktail to give final concentrations as given above for the rapid assay and by counting the trichloroacetic acid-precipitable material after filtration through membrane filters (Millipore Corp.). Assays at 32 and 39°C were performed by preincubation of the virus with 0.1 M Tris-hydrochloride, pH 7.5, at the indicated temperature for varying times followed by addition of the remaining components of the cocktail.

Immunoprecipitation of virus-specific proteins and gel electrophoresis of the proteins were as described previously (18).

RESULTS

A rapid screen for release of reverse transcriptase activity. The protocol for assaying individual clones of cells for released reverse transcriptase activity is shown in Fig. 1. Cells were infected with a preparation of wild-type M-MuLV and replated into cloning wells; after growth, part of the culture medium was added to a reverse transcriptase exogenous reaction mixture with detergent and incubated, and a portion was then spotted onto DEAE paper. The paper was washed and exposed to film for autoradiography.

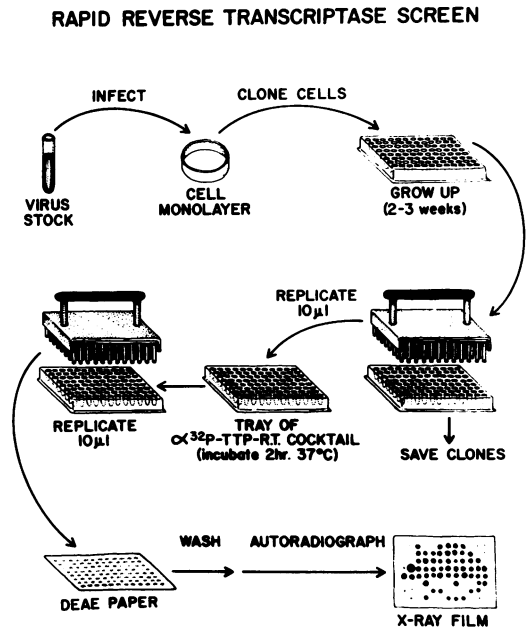

FIG. 1. *Outline of the procedure for assaying cloned cell lines for release of viral reverse transcriptase. Cells were infected with virus, cloned, and grown for 2 to 3 weeks. Ten microliters of the culture medium was removed and mixed with an enzyme cocktail. After incubation, the [^{32}P]DNA product was bound to DEAE paper, which was washed and exposed to X-ray film.*

A typical assay on 10 trays is shown in Fig. 2. In the experiment, approximately half of the wells contained cells (412 of 960); approximately one-third of these (125 of 412) were releasing reverse transcriptase enzyme. Duplicate assays of the same cells were reproducible (data not shown; see below). Although the number of cells in each well at the time of assay was highly variable, there was no obvious correlation between the number of cells and the intensity of the signal. Many of the very dark signals were produced by only a few cells ($<10^3$), whereas many of the faint signals were produced by confluent wells (containing 10^4 cells or more). We concluded that there was a wide range in the level of enzyme released by cell lines, as has been noted by others (15). The sensitivity of the assay was very high; as few as 100 cells produced positive signals. The assay shown was completed in a few hours of work, and the film shown was a 12-h exposure.

Isolation of mutants coding for thermolabile reverse transcriptase. To detect mu-

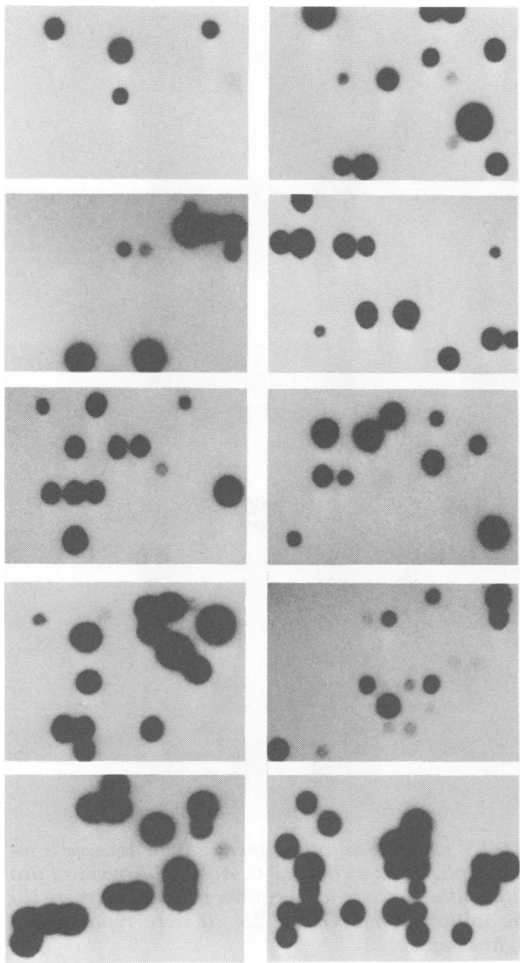

FIG. 2. *A typical assay of cloned cell lines for released reverse transcriptase. Ten trays of cells were prepared and assayed as outlined in Fig. 1. The [^{32}P]DNA bound to DEAE paper was exposed to X-ray film for 12 h.*

tants of M-MuLV which encoded a thermolabile reverse transcriptase activity, clones were prepared in 20 trays as described above and allowed to expand for 10 days at 37°C. The cells were then grown for 2 more days at 32°C. Portions of culture medium were removed and added in duplicate to an incomplete cocktail mix which lacked the template, primer, and nucleoside triphosphate. After preincubation for 1 h at 45 or 25°C, the template, primer, and dTTP were added, and the reaction was allowed to proceed for 2 h. An X-ray film exposure of two pairs of trays is shown in Fig. 3A. In general, the clones released temperature-resistant enzyme which showed similar activity at the two temperatures; a few, however, released enzyme with a much reduced activity at 45°C. A very few seemed to be cold sensitive. A total of 13 clones were selected (11 temperature sensitive and 2 cold sensitive), grown into larger cultures, and reassayed as described above. Of these 13, 10 were reproducibly thermolabile (Fig. 3B); their behavior was similar to that of the R-*ts*29, as previously described (17). No consistently cold-sensitive clone was detected.

Biological properties of the reverse transcriptase mutants. Each of the 10 putatively mutant clones was grown at 32°C, and each culture medium was harvested. The released virions were preincubated for 45 min at 32 or 39°C and then titrated in XC plaque assays performed at the same temperature. Of the 10, 5 never gave XC plaques under any condition and presumably were either uninfectious due to the mutation or were XC-negative variants of M-MuLV (5, 9, 10). Of the 10, 3 were XC positive, but showed no thermolability over that of the control wild-type virus (which lost 50 to 80% of its infectivity at 39°C, relative to 32°C, in this experiment). The XC titers of representative members of this group (TSP-6H3 and TSP-11F8) are shown in Table 1. The remaining two mutants (TSP-11A4 and TSP-6E9) were obviously more thermolabile than the wild type was in the XC assay; in separate experiments, they showed a 40- to 200-fold reduced titer at the higher temperature (Table 1). They also had lower titers than did the wild type after 32°C incubation. Thus, these two mutants, chosen only on the basis of the in vitro reverse transcriptase assay, were also thermolabile by plaque assay.

Kinetics of inactivation of reverse transcriptase. To confirm the qualitative results of the rapid assay, conventional reverse transcriptase assays were used. Virion harvests were collected from the mutant cell lines and preincubated for varying times in buffered medium at two temperatures, and then exogenous reverse transcription was allowed to proceed for 1 h. Three conditions were tested: (i) preincubation and assay at 25°C; (ii) preincubation and assay at 39°C; and (iii) preincubation at 39°C and assay at 25°C. Examples of inactivation curves for four of the mutants, along with control curves of wild-type M-MuLV and R-*ts*29, are shown in Fig. 4.

Wild-type viral reverse transcriptase was inactivated approximately 50% in this experiment during preincubation at 25°C; it showed some additional loss when preincubated at 39°C. In other experiments, the loss of activity was less but was generally 10 to 50% and was slightly

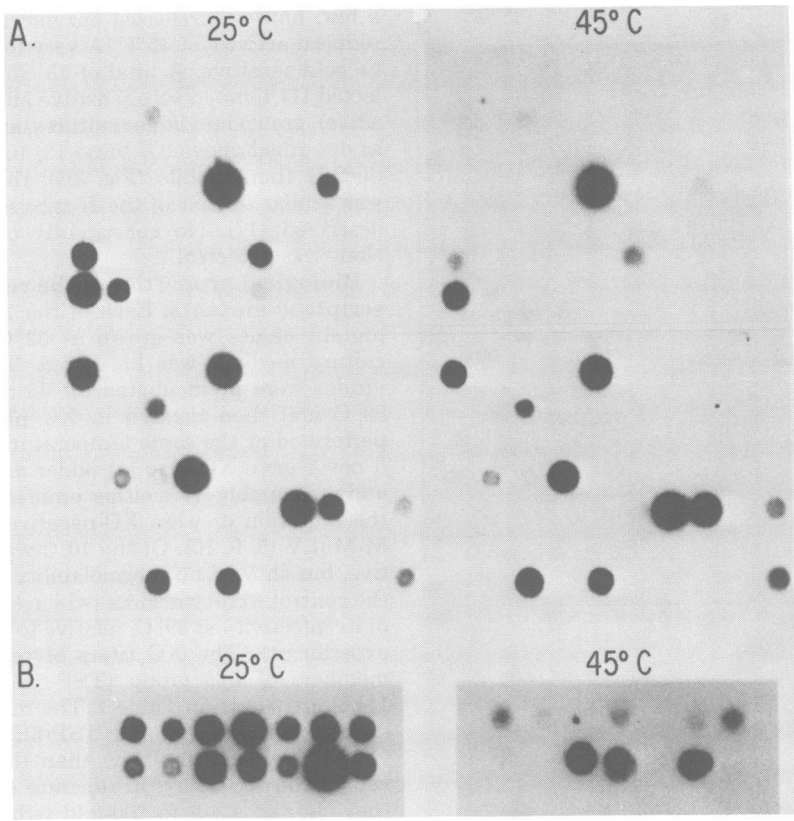

FIG. 3. *Isolation of cell clones containing mutant M-MuLV genomes encoding thermolabile reverse transcriptase. (A) Ten trays of cloned cells were assayed in the duplicate at 25 and 45°C. Autoradiograms of two of these trays are shown. A total of 13 clones showing differential activity were chosen from this screen. (B) Repeat of the assay on the 13 clones chosen from the initial screen. Of the 13, 10 were reproducibly thermolabile. R-ts29 is included for comparison (lower right sample).*

TABLE 1. *XC plaque assay on thermolabile reverse transcriptase mutants*[a]

Virus	Expt 1			Expt 2		
	Temp (°C)		Ratio (32°C/39°C)	Temp (°C)		Ratio (32°C/39°C)
	32	39		32	39	
Wild type	3×10^5	5×10^4	6	5×10^4	2×10^4	2.5
TSP-6H3	5×10^1	2×10^1	2.5	5×10^1	2×10^1	2.5
TSP-11F8	9×10^5	2×10^5	4.5			
TSP-6E9	5×10^3	$<5 \times 10^1$	100	4×10^3	2×10^1	200
TSP-11A4	2×10^3	5×10^1	40	2×10^3	2×10^1	100

[a] Virus harvests were collected at 32°C. The virus was preincubated at the indicated temperature for 45 min and then titrated at the same temperature. Table entries are titers in PFU/ml.

greater during preincubation at the elevated temperature. The activity, when assayed at 39°C, was considerably reduced, independent of the time of preincubation. R-*ts*29 activity was stable at 25°C, but extremely sensitive to preincubation at 39°; 1 h reduced the activity 20-fold to undetectable levels even when the assay was performed at 25°C. Essentially no activity could be detected in any assay carried out at 39°C.

The reverse transcriptase released by each of the mutant clones was more thermolabile than the wild-type enzyme. The enzymes produced by two of the clones (TSP-11A4 and TSP-6E9) were extremely temperature sensitive; like R-*ts*29, they were rapidly inactivated by preincubation at 39°C (Fig. 4). They also showed almost

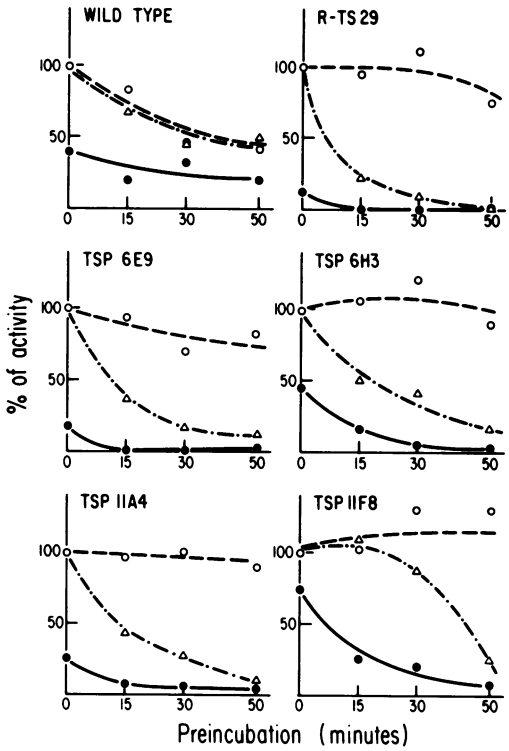

FIG. 4. *Heat-inactivation curves for reverse transcriptase released from wild-type M-MuLV-infected cells, R-ts29-infected cells, and four mutant clones. Culture medium from each clone was preincubated for varying times and then assayed for a fixed time (1 h). The conditions were: both preincubation and assay at 39°C (●); preincubation at 39°C and assay at 25°C (△); and both preincubation and assay at 25°C (○). Each of the four clones released a reverse transcriptase activity more thermolabile than that of the wild type.*

no activity when assayed at 39°C. Two others were less sensitive to preincubation, with activity falling slowly during preincubation and assay at 39°C. Clone TSP-11F8 produced enzyme which was particularly resistant to a 30-min preincubation at 39°C if the assay was then performed at 25°C. Thus, the enzyme from this clone was not irreversibly inactivated by the preincubation. It is noteworthy that the two viruses containing the most thermolabile enzymes were the same two which showed temperature sensitivity in the XC plaque assay (Table 2).

Associated phenotypic changes in temperature-sensitive reverse transcriptase mutants. Four of the mutant cell lines were tested for additional effects of the mutation on the assembly of virions. Cells were labeled overnight (at 32 or 39°C) with [^{35}S]methionine, and the virions released in that period were harvested and banded by isopycnic centrifugation. When the labeled proteins in the virions were analyzed by polyacrylamide gel electrophoresis (Fig. 5), cells infected with wild-type virus were found to have released large amounts of virion proteins at both temperatures; the most abundant protein by far was p30, a product of the *gag* gene. Neither clone TSP-6E9 nor clone TSP-6H3 was temperature sensitive for release of virions (data not shown), although both produced a thermolabile reverse transcriptase activity and one, in addition, was temperature sensitive for plaque formation (Table 2). Thus, reverse transcriptase mutants are not always affected in release. Two other clones, however, (TSP-11A4 and TSP-11F8) were temperature

TABLE 2. *Summary of properties of four thermolabile reverse transcriptase mutants*[a]

Virus	Rapid reverse transcriptase assay	XC assay	Reverse transcriptase heat inactivation	Virion release
TSP-6H3	*ts*[b]	+ (very low)	*ts*	+
TSP-11F8	*ts*	+	*ts*	*ts*
TSP-6E9	*ts*	*ts*	*ts*	+
TSP-11A4	*ts*	*ts*	*ts*	*ts*

[a] Data were taken from Table 1 and Fig. 3 through 5.
[b] *ts*, Temperature sensitive.

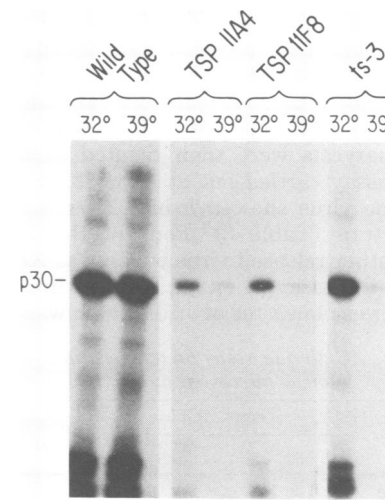

FIG. 5. *Analysis of virion proteins released into the culture medium by clones expressing thermolabile reverse transcriptase. Cells were labeled with [^{35}S]-methionine at 32 or 39°C, and the virions released into the medium were purified. The proteins were separated by electrophoresis through polyacrylamide gels and detected by fluorography. The position of the viral protein p30 is indicated.*

sensitive for release of virus (Fig. 5); much less p30 was detected in harvests at the high temperature. Their behavior was similar to that of M-MuLV *ts3* (Fig. 5). In a similar way, the R-*ts29*-infected line was temperature sensitive for release (data not shown). Thus, a number of independent reverse transcriptase mutants have been found to be, in addition, defective in their ability to assemble or release virions.

Isolation of mutants temperature sensitive for release of reverse transcriptase. The reverse transcriptase assay could also be used to detect and isolate mutations in retroviral proteins other than the reverse transcriptase itself. To detect clones with conditional mutations in any gene affecting assembly or release of virus, we chose to assay the reverse transcriptase released at each of two temperatures. Cells were infected as above and distributed to 10 trays of cloning wells. The clones were expanded for 20 days at 32°C, and the culture medium was assayed at 25°C (without a preincubation); then the medium over the clones was changed, and after 2 days at 39°C, a second portion was assayed at 39°C. Comparison of the two assays revealed approximately 11 clones (out of 200 producing reverse transcriptase) which released less activity at the elevated temperature; seven of these proved to be consistently temperature sensitive for release upon retesting. No consistently cold-sensitive clones were detected.

Preliminary characterization of mutants temperature sensitive for release. The virions of seven clones which were temperature sensitive for release were tested biologically. Culture medium was harvested from each of the seven clones after incubation for 1 day at 32°C. These harvests were then titrated in an XC plaque assay carried out at either 32 or 39°C. Wild-type virus showed a high titer at either temperature (Table 3). Four of the mutant clones either released virus which was XC negative in the assay at either temperature or released a very low titer of virus which was undetectable in our assays. The remaining three mutant clones released virions which were capable of producing plaques at 32°C but which produced no or fewer plaques when assayed at 39°C (Table 3). Thus, the three producer cell lines carrying these viruses were temperature sensitive for release of virion reverse transcriptase, and, moreover, the virus released at the permissive temperature was itself unable to complete an infection at the nonpermissive temperature.

TABLE 3. *XC plaque assay on temperature-sensitive virus harvested at 32°C*

Virus	Temp[a] (°C)		Ratio (32°C/39°C)
	32	39	
Wild type	4×10^5	2×10^5	2
TS 10C7	5×10^4	$<10^3$	>50
TS 1E6	8×10^4	3×10^3	27
TS 4D2	1.4×10^4	2×10^3	7

[a] Virus harvests were collected from cell lines after 1 day of growth at 32°C. The virus was preincubated at the indicated temperature for 45 min and titrated at the same temperature. Table entries are titers in PFU/ml.

The viral proteins synthesized by the seven cell lines were analyzed by metabolic labeling with [^{35}S]methionine, immunoprecipitation, and polyacrylamide gel electrophoresis. The products of the *gag* gene ($Pr65^{gag}$) and the *env* gene ($Pr80^{env}$) were detected in all of the clones; the only abnormality was a large overproduction of $Pr80^{env}$ at the elevated temperature by one of the XC-negative clones (TS 9F11) (Fig. 6). No other gross changes in the size or level of production of the viral proteins were detected among the mutants.

Isolation of mutants in the *gag* gene. Us-

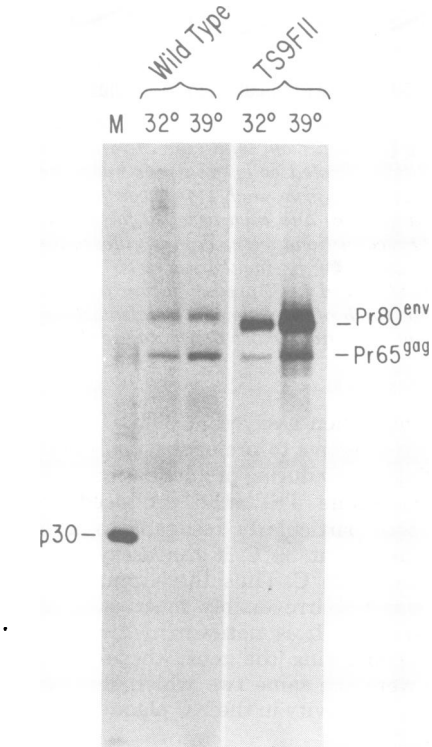

FIG. 6. *Envelope glycoprotein produced by the TS 9F11 clone. The indicated cells were labeled at 32 or 39°C with [^{35}S]methionine, and extracts were prepared. Viral envelope glycoprotein was immunoprecipitated. The proteins were analyzed by electrophoresis and fluorography.*

ing the rapid assay, clonal isolates of M-MuLV can be tested for their ability to replicate in two different host cells. If one of the hosts already carries a defective provirus which provides one of the viral functions, then mutants in that function would be detected as host range mutants, able to grow on the host carrying the function and not on normal cells. Hosts expressing the *env* function could not be used to isolate *env* mutants because they are not superinfectible, but hosts producing *gag* or *pol* proteins could, in principle, be used to isolate mutants in the *gag* or *pol* gene. One such host cell was available to us in the form of the cell line M23 (15), a derivative of NIH/3T3 cells carrying a defective M-MuLV provirus and synthesizing only $Pr65^{gag}$. Preliminary mapping (unpublished data) suggests that the provirus in M23 contains a 1.4-kb deletion mapping in the *env* gene, near the 3′ end of the viral genome. This cell line has been found to synthesize a short viral RNA (15) which apparently directs synthesis of only $Pr65^{gag}$; no *pol*- or *env*-related protein has been detected. Because this host cell is superinfectible, in principle it would be possible to isolate *gag* mutants with this host, which would grow by complementation but which would not replicate in normal cells.

Accordingly, M23 cells were infected with wild-type M-MuLV and distributed to 10 cloning trays as described above. After 15 days at 37°C, the cells were assayed for release of reverse transcriptase; at the same time, culture fluids were passed to fresh M23 cells and in duplicate to fresh NIH/3T3 cells. After 2 days, these cells were also assayed. Two clones were detected which released transcriptase activity and released virus which productively infected M23 cells but whose yield did not infect NIH/3T3 cells (Fig. 7).

Analysis of the viral proteins synthesized by the two clones showed no alterations from wild-type controls (data not shown). Analysis of the virus released from these clones by XC plaque assay, however, showed a distinct difference between the clones and controls and confirmed the host range phenotype of these two mutants (Table 4). The supernatants were titrated on two hosts: M23 cells and NIH/3T3 cells. Wild-type virus, as well as wild type virus grown on M23 cells, showed identical titers on these two hosts. The virus released by the two clones showed a high titer on M23 and a very low titer on NIH/3T3 cells (Table 4). In addition, the mutant plaque numbers on M23 cells gave a linear response to dilution; their plaque numbers on 3T3 cells, however, dropped off very rapidly with dilution of the stock, suggesting that multiple hits were required to initiate an XC plaque. The data were consistent with the notion that two genomes were present in the virus preparations and that both must infect the same cell to produce a plaque. The virus would yield a higher and linear titer on M23 cells because these cells already contained one of the required viruses as an endogenous provirus.

Isolation of transformed nonproducer cell lines. A common problem in the analysis of replication-defective transforming viruses is the isolation of a transformed cell clone which is free of helper virus. We made use of the rapid reverse transcriptase assay to facilitate this procedure. A mixed stock of Abelson MuLV with its helper M-MuLV was collected from a producer cell. NIH/3T3 cells were infected at a low multiplicity (0.7 PFU/cell; 0.15 focus-forming units per cell), trypsinized, and cloned in microtiter trays. After growth for 13 days, the clones were assayed for released reverse transcriptase activity. Those clones scored as negative for release of reverse transcriptase were then scored visually for morphological transformation. In this way, several transformed nonproducer lines were rapidly isolated. In one representative experiment, 4% of the total clones showed the desired phenotype.

DISCUSSION

The reverse transcriptase assay described is rapid and inexpensive; several thousand clones can be screened in a day by one person. The assay is remarkably sensitive, allowing detection of activity from as few as a 100 cells. The screen is directly useful for cloning viruses and for the isolation of transformed nonproducer clones from a mixed virus stock of a transforming virus and its helper. In addition, the screen is extremely helpful in the identification of variant clones producing temperature-sensitive or host range mutants of retroviruses. We have isolated a small number of mutants of three types; more of each class and of other classes could easily be isolated for further analysis.

A remarkable finding implicit in our successful isolation of these mutants is the high frequency of spontaneous mutations which arise in putatively wild-type stocks of M-MuLV. Thermolabile and temperature-sensitive mutants were found in screens of only 200 to 300 clones; the frequency of recovery of mutants was in the range of 3 to 5%. This high frequency of appearance of mutations has been noted previously (15). Some step in the life cycle of M-MuLV, and presumably many other retroviruses, must be highly mutagenic; likely candidates for this step are the transcription of the provirus into

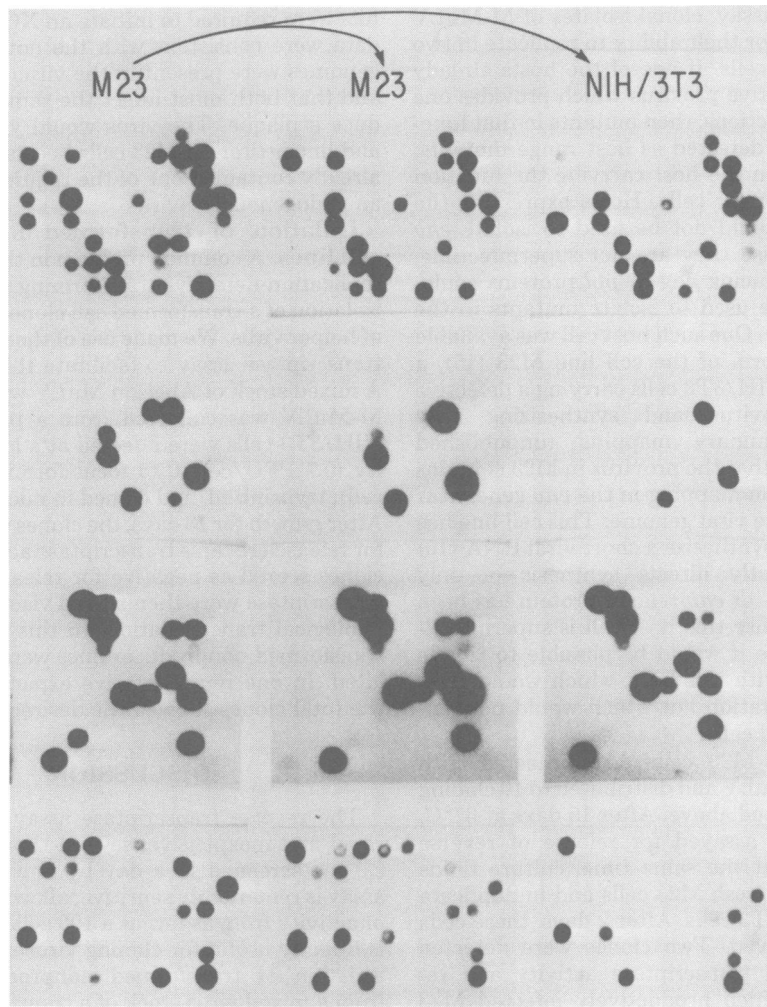

FIG. 7. *Isolation of host range mutants. M23 cells were infected with M-MuLV, cloned, and assayed for reverse transcriptase (left panels). The virus released by these cells was then passed to fresh M23 cells or to fresh NIH/3T3 cells. After 2 days, these cells were also assayed (middle and right panels). Clones releasing virus capable of infecting M23 cells but not NIH/3T3 cells were selected for further study.*

the genomic RNA and the reverse transcription of the RNA into circular double-stranded DNA. Both of these steps are thought to be carried out by nonproofreading polymerases and may well have high error rates. There may thus be several mutations introduced into each retrovirus genome each time it is passed from cell to cell.

The mutants isolated in our first screen are probably defective in the *pol* gene itself because the enzyme is thermolabile in vitro by two assays. It is nevertheless possible that mutations in adjacent viral proteins could reduce the stability of the enzyme. It is interesting that the *pol* mutants often, but not obligatorily, have defects in assembly and release of virions. These mutants raise the possibility that the polymerase protein or a precursor of the enzyme is involved in virion assembly at the cell surface as is also suggested by other data (P. Traktman and D. Baltimore, manuscript in preparation).

The mutants recovered in the second screen could potentially lie in any viral gene, or even potentially in a cellular gene, needed for assembly; the high frequency of appearance of these mutations would argue for a viral origin, and those that have a temperature-sensitive yield must be in the virus. The only gross alteration in a major viral protein was the overproduction of the glycoprotein $Pr80^{env}$ at the nonpermissive temperature in one clone. All of the other mu-

TABLE 4. *XC plaque assay on virus preparations harvested from cloned cell lines*[a]

Virus prepn	Dilution	Cell lawn			
		NIH/3T3		M23	
Wild type grown on M23 cells	1:100	TMTC[b]	TMTC	TMTC	TMTC
	1:300	500	500	500	500
	1:1,000	200	200	200	200
	1:3,000	100	100	100	100
	1:10,000	75	47	37	45
	1:30,000	22	16	12	15
	1:100,000	6	6	4	3
Clone 8G4	1:100	100	200	TMTC	TMTC
	1:300	23	13	500	500
	1:1,000	2	1	150	150
	1:3,000	2	2	64	—
	1:10,000	0	0	30	—
	1:30,000	0	0	6	—
	1:100,000	0	0	0	1
Clone M16	1:100	100	100	TMTC	TMTC
	1:300	13	17	300	300
	1:1,000			100	—
	1:3,000	0	0	43	40
	1:10,000	0	0	14	12
	1:30,000	0	0	4	—
	1:100,000	0	0	0	1
Wild-type virus grown on NIH/3T3 cells	1:1,000	105		57	
	1:10,000	13		4	
	1:100,000	1		0	
	1:1,000,000	0		0	

[a] Virus harvests from the indicated cell lines were diluted and used to infect either NIH/3T3 cells or M23 cells. Table entries are plaques counted per 6-cm dish.

[b] TMTC, Too many to count.

tants presumably either carry point mutations which inactivate the function of a particular protein without altering its size or alter undetected minor proteins.

Mutations of our third class, which are complemented by the defective provirus of M23 cells, presumably lie in the *gag* gene. In M23 cells these mutants replicate normally, but in NIH/3T3 cells they do not. Apparently recombination did not occur in the initial infection because in preliminary experiments two RNA sizes were still detectable in the cells: full-size RNA and the deleted M23 RNA (unpublished data). Similarly, among the rare successfully infected NIH/3T3 cells there were some reverse transcriptase-positive clones which also carried both RNAs. Thus both genomes can be released and apparently must be transmitted to a newly infected cell if that cell is to become a producer of virus. The virus thus shows a high titer on M23 cells, which already provides one of the needed genomes, and a very low titer on NIH/3T3 cells. The fact that the mixed virus stock shows multihit kinetics on NIH/3T3 cells suggests that both genomes rarely are packaged into one virion or, more likely, that both genomes in a virion are rarely converted to functional proviruses.

We are attempting to clone these mutants free of their M23 helper by screening NIH/3T3 cells infected at a low multiplicity for the synthesis of viral proteins. We can then analyze the mutants biochemically and determine the exact nature of the defect in the *gag* gene.

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