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Intracellular Restriction of Ecotropic Murine Leukemia Virus in Rat NRK Cells and Its Abolishment by Adaptation

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Ecotropic murine leukemia viruses, both N-tropic FN-2 (purified helper component of Friend leukemia virus) and B-tropic WNB-2 (purified WN1802B BALB/c-derived endogenous virus), were partially restricted in rat NRK cells. In NRK cells, they produced obscure small plaques at reduced efficiencies relative to their plaque-producing efficiencies in mouse SC-1 cells (10-fold for FN-2 and 100-fold for WNB-2). After three or four passages in NRK cells, the plaquing efficiencies of the viruses in NRK cells increased to levels close to their efficiencies in mouse cells, and the plaques in NRK cells became larger and clearer. The adaptation was more complete with FN-2 than with WNB-2. The adaptation was not due to simple selection of a virus in the FN-2 stock, but was host induced, as the viruses had been submitted to successive limiting dilutions in SC-1 cells before propagation in NRK cells. Possible commitment of xenotropic virus in the adaptation was excluded. The change was stable, even if the adapted viruses were propagated back into SC-1 cells. The NRK-adapted viruses were restricted in other rat cell lines of different origins, and the virus adapted in another rat cell line, RFL, was still restricted in NRK cells. The adaptation was mainly brought about by increased viral growth within the rat cells and not by an increased efficiency of viral penetration into the rat cells. This inversely suggests that the restriction of the ecotropic murine leukemia viruses in NRK cells was a mainly intracellular event. The mobilities of gp69/71 and p30 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis remained unchanged after adaptation of FN-2 in NRK cells.

Ecotropic murine leukemia viruses ($MuLV_E$), whose sensitive hosts are mice, are restricted partially in rat cells (see reference 7 for review). In general, restriction of $MuLV_E$ in nonmouse cells has been considered to be determined by their envelope characters; i.e., the restriction occurs at the level of viral penetration (2, 10). Restriction of $MuLV_E$ at the level of viral penetration in rat NRK cells has been documented (10). However, in avian leukosis viruses whose subgroup specificity was determined by the viral envelope, the restriction was complete (21), whereas intracellular restriction was partial, as in the case of restriction of induced subgroup E avian type C viruses in certain chicken cells (17) or Fv-1 restriction of $MuLV_E$ (3). From these points, we suspected that the partial restriction of $MuLV_E$ in rat cells might occur intracellularly. In this paper we describe experiments examining what viral changes were induced by adapting $MuLV_E$ to rat cells.

MATERIALS AND METHODS

Cells. Normal rat cells were Osborne-Mendel rat-derived NRK cells (3), Fischer rat-derived 3Y1 cells (11), and WKA rat-derived RFL cells (13). SC-1 cells, derived from a feral mouse (6), XC cells (12), and S+L- mink cells (16) were obtained from W. P. Rowe of National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, Md. Culture medium consisted of 9 parts Eagle minimal essential medium (Nissui Co.) and 1 part fetal calf serum inactivated by heating at 56°C for 30 min.

Virus. N-tropic Friend leukemia virus was obtained from the culture fluid of FV131 cells (22), and B-tropic WN1802B (14) were obtained from the culture fluid of an infected C57BL/6 cell line. Before starting the experiments, the viruses were subjected to two successive limiting dilutions in SC-1 cells. The virus stocks thus obtained were coded as FN-2 and WNB-2, respectively. The protocol of isolation is summarized in Fig. 1. Both FN-2 and WNB-2 were obtained from the dilutions which were 2 logarithmic units higher than the limit of plaque formation in the rat NRK cells.

NZB mouse-derived $MuLV_X$ propagated in mink cells (16) was obtained from A. Ishimoto of NIAID.

Virus titration. $MuLV_E$ was titrated by the standard UV-XC assay method (18), and $MuLV_X$ was titrated by the UV-S+L- mink method (10).

Analysis of viral proteins. Radioactively labeled virions were prepared by incubating the virus-producing cells for 24 h in leucine-free minimal essential

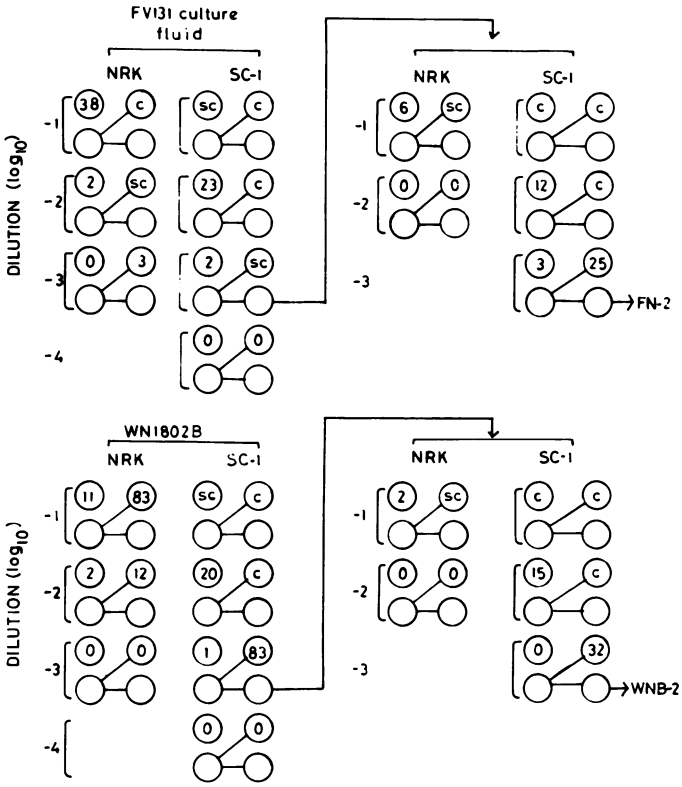

FIG. 1. *Cloning of MuLV$_E$ by limiting dilutions. N-tropic Friend leukemia virus produced by FV131 cells or B-tropic WN1802B was serially diluted 10-fold and used to infect SC-1 and NRK cells. One of the two dishes used for each dilution was submitted to the XC assay, and the other was subcultured for virus harvest. The culture fluid of SC-1 cells infected with the highest positive virus dilution (10^{-3} for both viruses) was further used for the next limiting dilution and infection. The culture fluid of the SC-1 cells infected with the highest virus-positive dilution in the second limiting dilution (10^{-3}, which was 100-fold higher than the limit of infectivity in NRK cells) was used as cloned virus. FV131-derived virus stock was coded as FN-2, and WN1802B-derived stock was coded as WNB-2. Each circle in the figure indicates a petri dish. The numbers in the circles indicate the numbers of XC plaques in the dishes (c = confluent; sc = semiconfluent). Empty circles indicate dishes used for virus harvest.*

medium containing 60 μCi of [^{3}H]leucine per ml and 10% fetal calf serum. The labeled virus was purified according to the method of Duesberg et al. (4). The purified virions were heated at 100°C for 2 min with 1% sodium dodecyl sulfate and 5% β-mercaptoethanol and subjected to electrophoresis in 8 to 15% polyacrylamide gel in a discontinuous system (14). The electropherogram was visualized by staining with Coomassie brilliant blue and fluorography.

RESULTS

Propagation of FN-2 and WNB-2 in NRK cells. FN-2 and WNB-2 were titrated in NRK and SC-1 cells by the standard UV-XC procedures (18). Restriction in NRK cells was observed in the following two points: (i) plaque size (see Fig. 3) and (ii) plaquing efficiency. To give an approximate idea of plaque size, we scored plaques at magnifications of ×15 and ×35 and calculated the ratio (15/35 ratio). If plaques were small and obscure, they were not scored at the low magnification. Thus, dishes containing many such small plaques showed low 15/35 ratios. To compare plaquing efficiencies in NRK and SC-1 cells, we calculated the ratio of the titer (counted at magnification of ×35) in NRK cells to the titer in SC-1 cells (N/S ratio).

The N/S ratios were 0.02 to 0.09 for FN-2 and 0.002 to 0.015 for WNB-2. The 15/35 ratios in NRK cells were 0.05 to 0.3 for FN-2 and 0.2 to 0.4 for WNB-2; the ratio was 1 in SC-1 cells for both FN-2 and WNB-2 (Fig. 2, S).

One of the NRK cultures infected with the highest virus dose was subcultured, and the culture fluid was harvested for the next infection. This procedure was repeated. N/S and 15/35 ratios were estimated for virus of each NRK passage (Fig. 2, N1 through N4). After each passage, both the 15/35 ratio in NRK and the N/S ratio increased, attaining plateaus at the

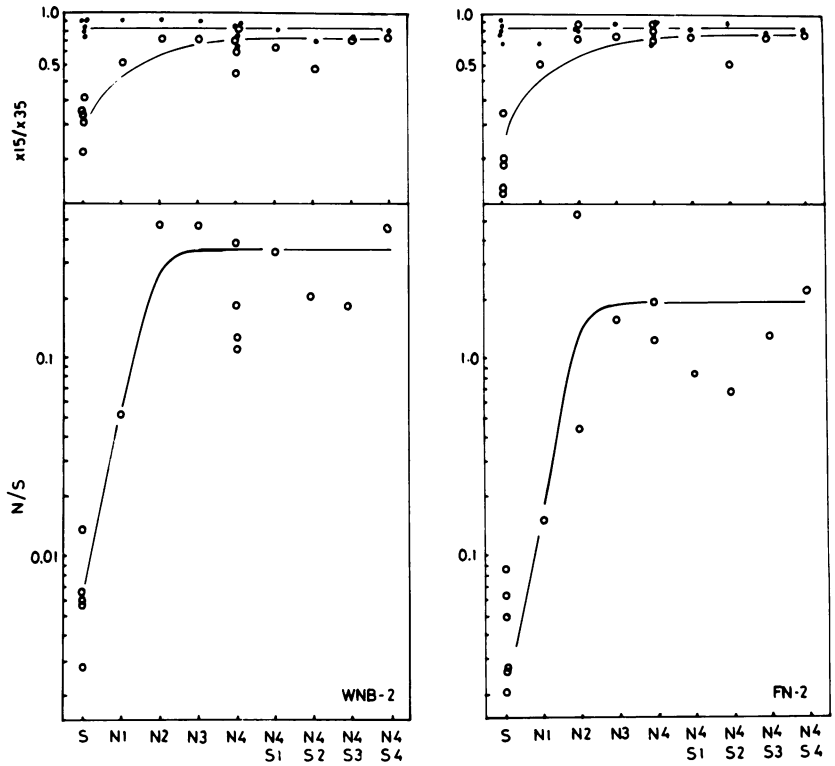

FIG. 2. *Adaptation of FN-2 (right) and WNB-2 (left) in NRK cells. FN-2 or WNB-2 was used to infect SC-1 and NRK cells. The culture fluid of NRK cells infected with the highest virus dose was used for the next cycle of infection. The procedure was repeated. After four passages in NRK cells, the viruses were propagated back into SC-1 cells four times. Each passage was tested for the 15/35 ratios in SC-1 and NRK cells and the N/S ratio. Abscissa: (S) FN-2 or WNB-2 propagated in SC-1; (N1 through N4) passages 1 through 4, respectively, in NRK cells; (N4S1 through N4S4) passages 1 through 4 in SC-1 cells of the virus which had been propagated four times in NRK cells. Ordinate: (×15/×35) number of plaques counted at 15-fold magnification divided by number of plaques counted at 35-fold magnification in SC-1 cells (●) and in NRK cells (○); (N/S) plaquing efficiency in NRK cells divided by plaquing efficiency in SC-1 cells (plaques were counted at a magnification of ×35).*

third passage. The N/S ratios were from 1 to 2 for FN-2/N4 (FN-2 passaged 4 times in NRK) and about 0.2 for WNB-2/N4 (WNB-2 passaged 4 times in NRK).

Figure 3 shows clear and large plaques of FN-2/N4 in NRK cells, in contrast to obscure plaques of FN-2 in the same cells. In SC-1, both viruses produced plaques of similar size.

Figure 4 compares the growth curves of FN-2 and FN-2/N4 in NRK and SC-1 cells. Though FN-2/N4 replicated with the same kinetics in NRK and in SC-1 cells, the growth curve of FN-2 in NRK was shallow, in contrast to the steep growth curve in SC-1.

To examine the genetic stability of such adaptation, we propagated FN-2/N4 and WNB-2/N4 back into SC-1 cells (Fig. 2, N4S1 through N4S4). After four successive passages in SC-1 cells, the 15/35 and N/S ratios remained unchanged. Thus, the host-induced changes produced in FN-2 and WNB-2 were stable.

Role of MuLV$_X$ in the adaptation. As both FN-2 and WNB-2 were submitted to the two successive limiting dilutions in SC-1 cells and were obtained from the cells infected with the virus diluted 2 logarithmic units higher than the limit of plaque formation in NRK cells, the adaptation was not due to a simple selection of NRK-nonrestricted virus which already existed before NRK passage. The adaptation seemed to be initially induced by a host modification, followed by selection.

However, we examined whether NRK-adapted viruses had xenotropic characters or not. For this purpose, FN-2/N4 or WNB-2/N4 was first used to infect NRK cells; 4 days later, the cultures were subjected to the UV-S+L− mink assay (10). No transformation was observed. Apparently these NRK-adapted viruses contained no xenotropic virions. We also tested

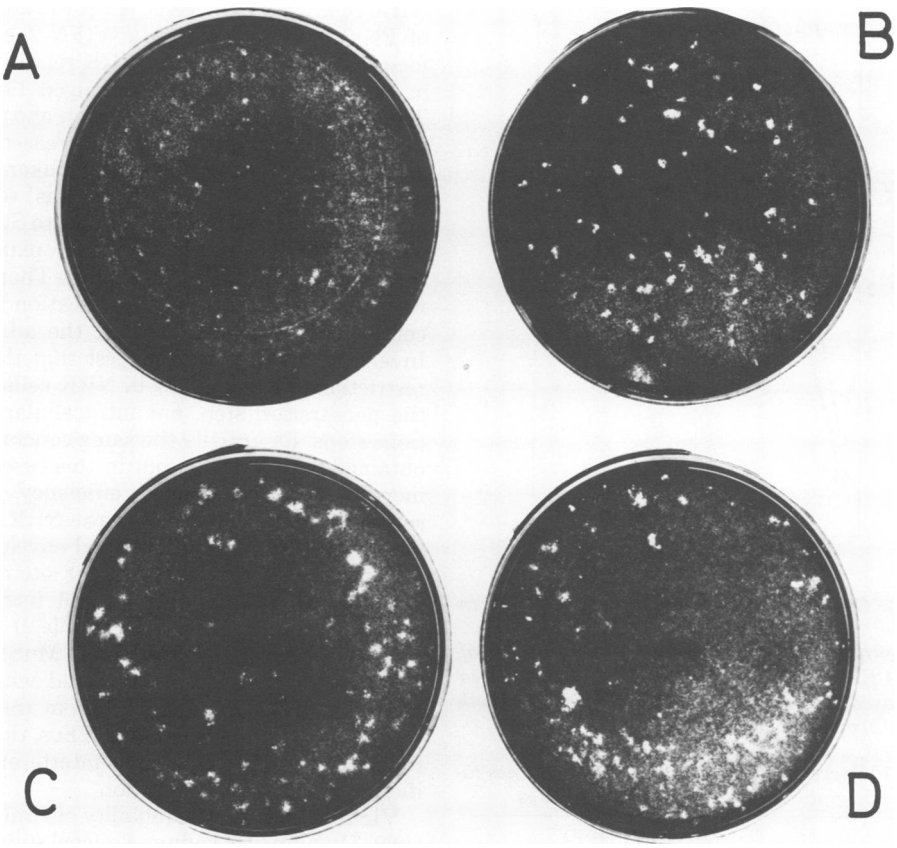

FIG. 3. *XC plaques of FN-2 and FN-2/N4 in NRK and SC-1 cells, scored at a high magnification (×35). (A) NRK infected with FN-2; 174 plaques were scored. (B) NRK infected with FN-2/N4; 86 plaques were scored. (C) SC-1 infected with FN-2; 96 plaques were scored. (D) SC-1 infected with FN-2/N4; 102 plaques were scored. Note that (A) contained twice as many plaques as (B) when the plaques were counted at a high magnification.*

the possibility that prior infection with MuLV$_X$ might result in an enhanced plaquing efficiency of MuLV$_E$ in NRK cells. For both NRK and SC-1, MuLV$_X$-infected cells were two- to fourfold more sensitive to MuLV$_E$ than were control uninfected cells (data not shown). However, MuLV$_X$ infection never increased MuLV$_E$ infectivity in NRK cells to the level observed in the case of repeated passages of MuLV$_E$ in NRK cells. From these experiments, involvement of MuLV$_X$ in the adaptation was excluded.

Sensitivity of other rat cells to MuLV$_E$ before and after NRK adaptation. WKA rat-derived RFL and Fischer rat-derived 3Y1 cells were tested for sensitivity to control and NRK-adapted viruses. As shown in Table 1, the NRK-adapted as well as the original viruses failed to plate efficiently on RFL and 3Y1 cells. On the other hand, FN-2/R3, which was obtained by three serial passages of FN-2 in RFL cells in the same experimental protocol as that used for NRK adaptation, plated on NRK cells at a relatively low efficiency. These observations may suggest a host specificity of adaptation.

The tropism of viruses with respect to *Fv-1* (8) remained unchanged after the adaptation (data not shown).

Level of NRK adaptation of MuLV$_E$ in the infection cycle. We examined which level of viral replication in NRK cells was facilitated by adaptation, i.e., the level of viral penetration or the level of intracellular replication. Murine sarcoma virus (MuSV) appears to require no virus propagation for focus formation in NRK cells (20). Thus, focus formation in NRK cells is mainly determined by the viral penetration, and replication of the virus is not mandatory. The MuLV$_E$s before and after adaptation in NRK cells were used to rescue MuSV from S+L− C182 cells (1) whose MuSV genomes were lacking in gp69/71 (and also in polymerase) (15). The rescued MuSV, thus enveloped in the coat of MuLV$_E$, were titrated in SC-1 and NRK cells. MuSV(FN-2) produced foci as efficiently in

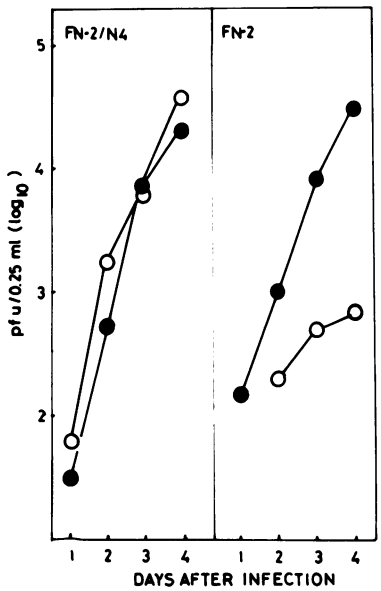

FIG. 4. *Growth curve of FN-2 and FN-2/N4 in NRK and SC-1 cells. NRK and SC-1 cells were infected with FN-2 or FN-2/N4 at a multiplicity of about 0.1 Every day, 24-h culture fluid was harvested and assayed in SC-1 cells. Symbols: ●, virus growth in SC-1; ○, virus growth in NRK.*

TABLE 1. *Susceptibilities of various rat cell lines to the original and NRK-adapted MuLV$_{ES}$*

Virus	Cell sensitivity[a]		
	NRK	RFL	3Y1
FN-2	11	3	7
FN-2/N4	271	1	24
FN-2/R3	36	250	14
WNB-2	2	0.3	0.2
WNB-2/N4	77	0.6	0.5

[a] MuLV$_{ES}$ were titrated by the standard UV-XC procedures. The sensitivities are expressed as the percent sensitivity relative to SC-1 cells.

NRK cells as in SC-1 cells. After the adaptation of FN-2 in NRK cells [MuSV(FN-2/N4)], the focus-forming efficiency in NRK relative to that in SC-1 cells remained unchanged (Table 2). This indicates that the adsorption and penetration into NRK cells was not increased by the adaptation. On the other hand, leukemia virus coexisting with MuSV(FN-2) was restricted about 10-fold in NRK cells relative to SC-1 cells, whereas that in MuSV(FN-2/N4) plated even better in NRK than in SC-1 cells. Therefore, it was the intracellular viral replication in NRK cells which was facilitated by the adaptation. Inversely, these results suggest that the major restriction site of MuLV$_E$ in NRK cells was not the penetration step, but intracellular replications steps. Essentially the same conclusion was obtained with WNB-2, but in this case a slight increase in focus-forming efficiency was observed with MuSV rescued by the NRK-adapted virus. Thus, changes in the viral envelope were not excluded here, but the major site of alteration by the adaptation was still intracellular replication in the NRK cells (Table 2).

Both MuSV(FN-2/N4) and MuSV(WNB-2/N4) were completely interfered with by an MuLV$_E$, WN1802B, obtained from the laboratory of W. P. Rowe at NIAID. Thus, the viruses remained unchanged in their interference specificities after NRK adaptation.

Viral proteins. The mobility of viral proteins gp69/71 and p30 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis was examined for FN-2, FN-2/N1, FN-2/N5, and FN-2/N4S1. Actually, no detectable changes were produced by the adaptation (Fig. 5).

DISCUSSION

Our experiments demonstrated that MuLV$_{ES}$ were restricted in the rat NRK cells, and successive passages of the virus in NRK cells resulted in a stable adaptation which was manifested in

TABLE 2. *Infectivity of MuSV pseudotypes in SC-1 and NRK cells*

Expt	Cell line	Virus							
		MuSV (FN-2)		MuSV (FN2/N4)		MuSV (WNB-2)		MuSV (WNB-2/N4)	
		MuSV (FFU/0.25 ml)[a]	MuLV (PFU/0.25 ml)[b]	MuSV (FFU/0.25 ml)	MuLV (PFU/0.25 ml)	MuSV (FFU/0.25 ml)	MuLV (PFU/0.25 ml)	MuSV (FFU/0.25 ml)	MuLV (PFU/0.25 ml)
1	SC-1	1,000	2,600	890	3,200	600	1,160	430	560
	NRK	1,110	230	1,100	2,400	83	5	180	260
	N/S[c]	1.11	0.09	1.25	0.75	0.14	0.004	0.42	0.46
2	SC-1	1,100	2,800	2,500	2,500	600	900	800	1,500
	NRK	1,100	800	2,800	12,000	99	6	460	216
	N/S	1.00	0.28	1.12	4.8	0.17	0.007	0.57	0.14

[a] Focus titration (FFU, focus-forming units).
[b] Plaque titration by the XC assay method (PFU, plaque-forming units).
[c] N/S, Plating efficiency in NRK cells divided by plating efficiency in SC-1 cells.

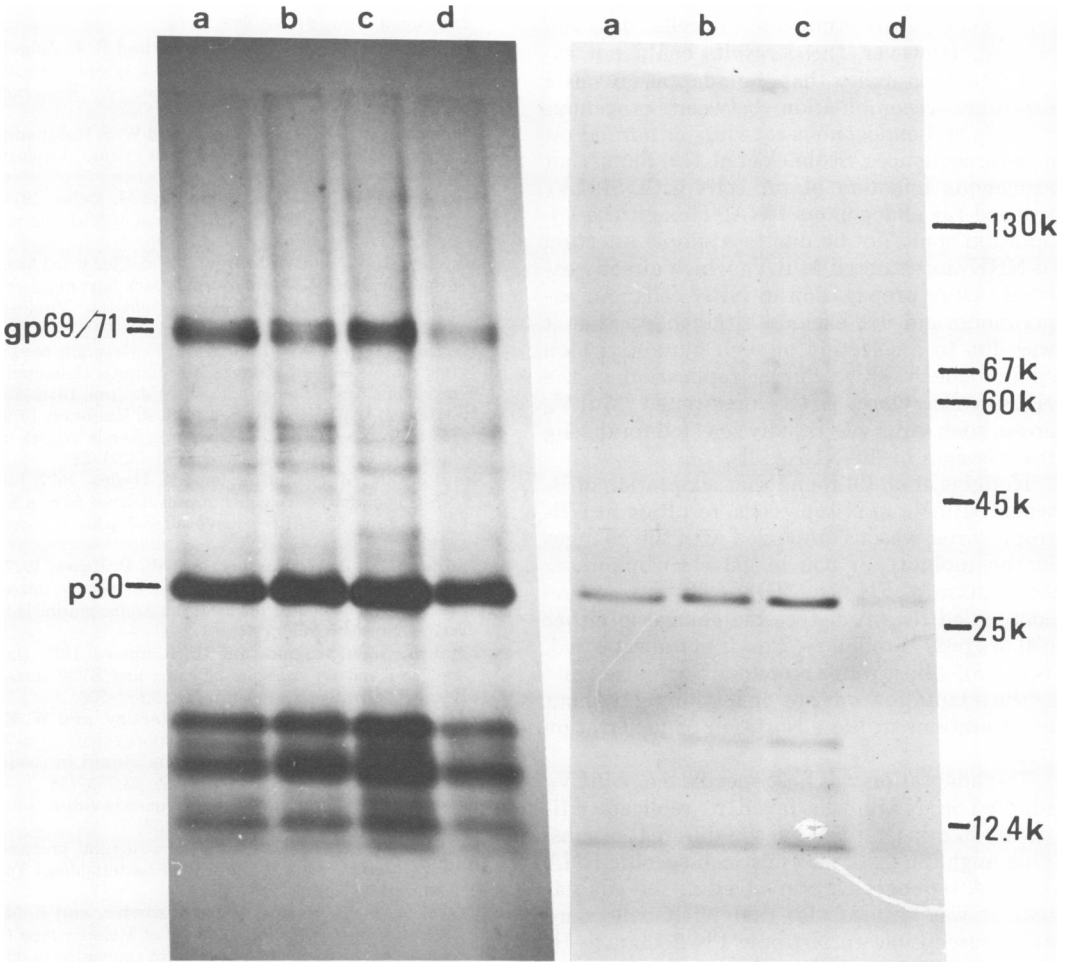

FIG. 5. *Polyacrylamide gel electropherogram of viral proteins visualized by fluorography (left) and by staining with Coomassie brilliant blue (right). Purified viruses labeled with [^{3}H]leucine were disrupted with 1% sodium dodecyl sulfate and β-mercaptoethanol and subjected to electrophoresis in 8 to 15% gradient polyacrylamide gels. (a) FN-2 virus released from SC-1 cells; (b) FN-2/N4S1 released from SC-1 cells; (c) FN-2/N1 released from NRK cells; (d) FN-2/N5 released from NRK cells. Standard proteins used as molecular markers were: 130K, β-galactosidase; 67K, bovine serum albumin; 60K, catalase; 45K, ovalbumin; 25K, chymotrypsinogen A; 12.4K, cytochrome c.*

the increased plaquing efficiency and formation of clear and large plaques in NRK cells. The adaptation seemed to occur mainly at the level of intracellular growth of MuLV$_E$ in NRK cells, and alteration of the viral envelope, if it ever occurred by the adaptation, played a minor role in the process. Actually, no changes of gp69/71 were detected in their molecular size in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These facts inversely indicate that major restriction site of MuLV$_E$s in NRK cells was not penetration but was intracellular growth, though the former role may not be excluded completely (2). A similar conclusion was obtained in the case of restriction of rat XC cells to MuLV$_E$ infection (5). These observations have close similarities to that described by Robinson (17) on the partial restriction of induced group E avian leukosis virus in certain chicken cells; namely, the restriction was intracellular and adaptation was fairly easily accomplished after a few passages of the virus in the resistant host cells. She noticed also that the adaptation occurred without any detectable changes in the viral envelope.

Possible involvement of MuLV$_X$ in the adaptation was excluded. However, our experiments on viral RNA size and homology in RNA sequence failed to elucidate the mechanism of the adaptation; the preliminary hybridization experiments between viral complementary DNA and cellular RNA failed to detect any increase in the sequence homologous to RNA from normal or

endogenous virus-producing rat cells (data not shown). However, these results could not exclude the possibility that the adaptation was a result of recombination between exogenous $MuLV_E$ and endogenous rat virus or normal rat genetic sequences. Scolnick et al. (19) found that exogenous infection of rat cells with $MuLV_E$ rescued rat endogenous RNA. Though the adaptation could not be due to a simple selection of NRK-unrestricted $MuLV_E$ which already existed before propagation in NRK cells, our experiments did not exclude a possibility that it was due to a selection of such mutants which spontaneously arose during replication in the NRK cells. Once NRK-unrestricted $MuLV_E$ arose, such virus was rapidly selected for during the passages in the NRK cells.

Hopkins et al. (9) found that adaptation of B-tropic $MuLV_E$ in N-type cells, resulting in NB-tropic virus, was accompanied with the changes in the mobility of p30 in gel electrophoresis. Adaptation of FN-2 to NRK cells was not accompanied by any detectable change in either p30 or gp69/71 mobility. This is not unexpected, as the *Fv-1* host range remained unchanged and as the adaptation was not mainly brought about by alterations in the adsorption or penetration steps.

The adaptation was host specific, i.e., $MuLV_E$ adapted in NRK cells failed to replicate efficiently in other rat cell lines of different origins. This might suggest that host cell-specific RNA or DNA sequences are involved in the adaptation. It was noticed also that NRK cells were more susceptible to N-tropic FN-2 than to B-tropic WNB-2, and the adaptation was more complete with the former. We have not yet tested whether this phenomenon was due to the presence of *Fv-1*-related restriction in the rat species or not, but, in view of relatedness between rats and mice and also of the surprisingly high conservation of genetic traits during evolution, the search for *Fv-1*-related restriction in rat species may be promising.

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