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Transdominant Inhibition of Moloney Murine Leukemia Virus Proliferation by Defective Mutants of Reverse Transcriptase

Yukio Shirasawa, Sumie Sato, Toshio Osawa, and Akikazu Hirashima[1]

Yakult Central Institute for Microbiological Research, Kunitachi, Tokyo 186

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To investigate the dominant-negative inhibition of Moloney murine leukemia virus (Mo-MuLV) proliferation by polymerization-defective mutants of reverse transcriptase (RT), we constructed several plasmids harboring the Mo-MuLV RT gene in which the YVDD sequence, one of the conserved sequences in RNA-dependent polymerases, was altered, and then transformed mouse NIH3T3 cells and *Escherichia coli* with the mutant plasmids. Mouse NIH3T3 cells expressing these mutant RT genes were highly resistant to Mo-MuLV proliferation. The mutant RT expressed in *E. coli* exhibited no polymerization activity, but it retained its binding activity to the template RNA and inhibited *in vitro* poly(dG) synthesis occurring with the wild-type RT. These results suggest that the competition for binding of the two types of enzymes to the template is responsible for the resistance to Mo-MuLV proliferation and that the YVDD sequence of Mo-MuLV may be a good target for dominant-negative inhibition of retroviral proliferation.

Key words: dominant-negative inhibition, intracellular immunization, moloney murine leukemia virus, reverse transcriptase, YXDD motif.

As proposed by Baltimore (*1*), "intracellular immunization" is regarded as genetic alteration of cells that is mediated by gene transfer and leads to the inhibition of viral replication. One of the strategies for intracellular immunization is dominant-negative mutation of the essential gene for viral replication. This strategy involves inhibition of the function of a wild-type gene product by an overproduced inhibitory variant of the same product (*2*). Such inhibitory variants of wild-type products can be designed if the proteins have multi-functional sites that can be mutated independently. In the same year as Herskowitz reported this technology (*2*), Inokuchi and Hirashima demonstrated that *Escherichia coli* cells expressing defective mutants of replicase were rendered resistant to RNA coliphage Qβ (*3*). Soon after, many investigators reported the inhibition of eukaryotic viral proliferation by modification of essential genes for viral replication and suggested that the dominant-negative inhibition strategy was a hopeful approach for prevention of eukaryotic viral diseases (*4-15*).

Reverse transcriptase (RT) of Mo-MuLV has a highly conserved YXDD sequence motif, in which X can be any amino acid residue (*16, 17*). Since this motif is found in numerous viral RTs, RNA polymerases and some DNA polymerases (*17, 18*), the strict conservation of the motif suggests an important role in the polymerase function. Several studies have revealed polymerases mutated in this conserved motif (*19-26*). For instance, mutations at the first aspartic acid (D) of the YXDD motif resulted in enzymes with drastically reduced activities (*19, 20*). Although mutations of the second and least-conserved residues of YXDD have produced enzymes with various levels of activity (*21-24*), amino acid substitutions at this position resulted in a significant change in divalent metal ion preference (*18, 24-26*), indicating that the YXDD motif constitutes a part of the active center of RT. In addition, in spite of the fact that RT activity was abolished by mutations of the YXDD motif, those mutations hardly affected the binding of enzymes to template RNA (*20, 25*). These results therefore led to the idea that intracellular immunization against viral infection can be performed through expression of a dominant-negative mutant of the polymerase gene. Indeed, this idea was first substantiated by Inokuchi and Hirashima for bacteriophage proliferation (*3*), and more recently by Zaitlin *et al.* and Longstaff *et al.* for plant viral diseases (*13-15*). However, to our knowledge, no analogous case of an animal virus has been reported so far. We therefore used the YXDD sequence of Mo-MuLV to examine the dominant-negative inhibition of retroviral proliferation.

We show here that (i) mouse cells expressing mutant RT genes became resistant to Mo-MuLV proliferation, and (ii) mutant RT which has no polymerization activity inhibited *in vitro* cDNA synthesis occurring with the wild-type RT.

MATERIALS AND METHODS

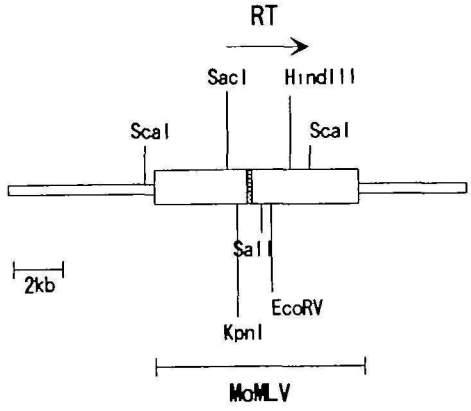
Plasmids and Mutagenesis—A plasmid, pMLV48, harboring infectious proviral DNA of Mo-MuLV (*27*), as shown in Fig. 1, was a gift from Y. Kuchino (National Cancer Center, Tokyo). pRT-T1/N harboring mutant RT gene of Mo-MuLV (*24*) was a gift from K. Saigo (the University of Tokyo). A shuttle vector which expresses the wild-type RT gene, pSM51, was constructed by ligating a *Sca*I-*Sca*I (6.5 kb in size) restriction fragment containing the LTR-gag-pol genes of pMLV48 to the restriction

[1] To whom all correspondence should be addressed.

fragment of pSV β-globin (*28*). For oligonucleotide-directed mutagenesis, a *Sac*I-*Hin*dIII fragment (2.3 kb in size) of the polymerase (pol) gene in pMLV48 or pRT-T1/N was re-cloned into coliphage M13, which is designated as M13-pol. Single-stranded DNA of M13-pol was prepared after infection of transformed *E. coli* CJ236 with an M13 helper phage, KO7 (*29*). The synthetic complementary deoxy-oligonucleotides shown in Fig. 1 were made according to the sequence of the mutation sites of RT. Site-directed mutagenesis was performed under the conditions described by the manufacturer of the site-directed mutagenesis kit, Mutan-K (Takara Shuzo). Candidate clones were screened for the RT gene without polymerization activity and the mutated sequences were confirmed by the dideoxy sequencing method. These mutants were designated together as M13-pol*. To express mutagenized RT in mouse cells, a *Sac*I-*Sal*I fragment (1.16 kb) from pSM51 was replaced by the corresponding fragment of M13-pol*, which was designated as pSM51-pol*, but represents pSM51-N17, -M151, -M153, and -M134 in this paper. These plasmids were used for transformation of NIH3T3 cells and the transient infection experiments.

To construct an RT overexpression plasmid in *E. coli*, pQE70, which can fuse to a tag consisting of 6 histidine residues at the carboxyl end of the target protein, was purchased from Qiagen. Since Kotewicz *et al.* reported that the RT gene-lacking RNase H region overexpressed active RT in *E. coli* cells (*30*), we adopted the following strategy to obtain the RT gene for overexpression. Namely, the wild-type RT gene was synthesized by PCR using pSM51 DNA as a template and two appropriate primers to create a new *Sph*I site at the 5′ end of the RT gene, and to create an *Eco*RV site at the 3′ end, which is located near nucleotide number 4085 in the Mo-MuLV genome (*31*). A *Sph*I-*Eco*RV fragment (1.45 kb) was ligated to the cloning sites for *Sph*I and *Eco*RV of pQE70, the resulting plasmid being designated as pQE70RT. To construct a plasmid harboring the mutant RT gene, a *Kpn*I-*Sal*I fragment (840 bases) of pQE70RT was replaced by the corresponding restriction fragment from pSM51-M153, the resulting plasmid being designated as pQE70RTM153. *E. coli* JM109 cells were transformed with these plasmids. One transformant, designated as QE70RT, produced a large amount of polymerization-active RT, whereas another, designated as QE70RTM153, produced a large amount of polymerization-inactive RT, when they were grown in the presence of 1 mM isopropyl-β-D-thiogalactoside (IPTG). Thus, we grew these strains as the source materials for purification of wild-type and mutant RTs used in the experiments of Fig. 4 and Table IV.

Cells, Viruses, Transfection, and Viral Infection—Mouse NIH3T3 cells were obtained from Riken Cell Bank (Tsukuba Science City) and grown in Dulbecco's modified



Mutant	Amino acid sequence	Substitution
WILD	LQYVDDLL	
N17	LQY**N**DDLL	V → N
M151	LQYV**F**DLL	D → F
M153	LQYV**T**DLL	D → T
M134	LQYVDDL**A**	L → A

Fig 1 **Restriction sites in the Mo-MuLV gene and the strategy for constructing mutant RTs.** Relevant restriction sites of pMLV48 (*27, 37*) are shown (Top) Using these restriction sites, various plasmids were constructed For example, *Sac*I and *Hin*dIII for M13-pol, *Sac*I and *Sal*I for pSM51-pol*, *Sph*I (introduced by PCR at the 5′ end of RT) and *Eco*RI for pQE70RT, and *Kpn*I and *Sal*I for pQE70RTM153 were used The dotted box indicates the position of the conserved YXDD region with an expanded sequence (LQYVDDLL), using a single-letter code for amino acids. For site-directed mutagenesis, single-stranded M13-pol DNA was annealed with the synthetic oligonucleotides Oligonucleotides were synthesized with an Applied Biosystem apparatus. Mutagenic 30-mers, GCCCAGCAGT AAGTCATCATTGTACTGTAG for N17 mutant, GCCCAGCAGTAA-GTCAAACACGTACTGTAG for M151 mutant, GCCCAGCAGTAA-GTCAGTCACGTACTGTAG for M153 mutant, and GCCCAGCGCT-AAGTCATCCACGTACTGTAG for M134, were of negative polarity with respect to the translation frame of the RT gene. Underlined nucleotides represent mispaired sequences for introducing single amino acid changes around the YVDD motif Mutagenesis was carried out as described in the text. Verification of the construction was carried out by direct dideoxy sequencing of the resulting plasmids. Mutants N17, M151, M153, and M134 refer to the mutations on substitution of V to N, D to F, D to T, and L to A, as indicated by underlining in this figure, respectively

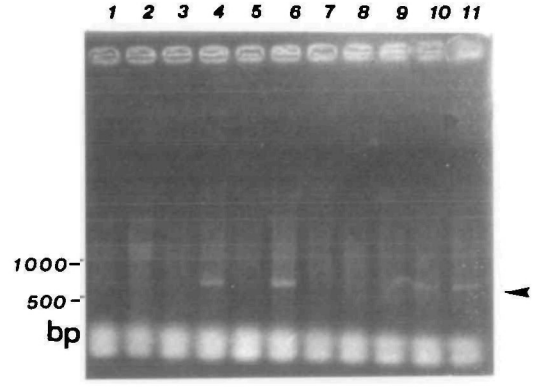


Fig 2. **Detection of mRNA for the RT gene expressed in the transformed cells.** Transformed cell clones which express RT gene were selected by an RT-PCR method Using two primers covering the coding sequence of RT gene (2601-3400 bases), a 799 bp long specific fragment (indicated by an arrowhead) was amplified and detected on a 1% agarose gel (lanes 4, 6, 10, 11) Lanes 1-5 represent G-418-resistant cell clones transformed with pSM51-N17. Lanes 6-9 show G-418-resistant cell clones transformed with pSM51-M151. Lane 10 and lane 11 represent cell clones which express the mRNAs for M153 and M134 RT genes, respectively Lanes 1, 2, 3, 5, 7, 8, 9 show that these cell clones did not have RT genes derived from the mutant plasmids.

Eagle's medium (DMEM) containing 10% fetal calf serum. Fresh Mo-MuLV stocks were prepared from the supernatant of NIH3T3 cells infected with Mo-MuLV.

To assess the interference by Mo-MuLV infection in the transient infection system, NIH3T3 cells were co-transfected with pMLV48, which generates progeny Mo-MuLV particles, and either the wild-type or a mutant RT gene-harboring plasmid in the mixing ratio of 1:2. At various times after transfection, the supernatant of withdrawn cultured medium was used to monitor the production of infectious particles (*32*).

To examine the resistance of cells harboring RT gene to viral infection, NIH3T3 cells were transformed with the plasmids constructed above and pIPB1, which consists of the neomycin phosphotransferase gene conferring resistance to the antibiotic, G-418, by electroporation. Then, G418-resistant clones were selected on 0.25 mg/ml of G-418 (GIBCO). Expression of mRNA for RT was detected by means of RT-PCR as shown in Fig. 2. Transformants which had grown on G-418 and which expressed the RNA were designated as N-17, M151, M153, and M134, respectively. These cells grew as well as the wild-type cells, and their resistance to Mo-MuLV proliferation was examined. The Mo-MuLV infection of these cells was carried out on 50-mm plates of 25-50% confluent cells with 8 μg/ml of Polybrene (Aldrich). At various times after infection, 5 ml of the supernatant of the harvested culture medium was used for the progeny formation assay (*32*). To measure the RT activity of the viral particles released from Mo-MuLV-infected cells, Mo-MuLV infection was carried out as described above except for the use of 10 ml of culture medium. At 7 days after infection, the supernatant was used to measure cDNA synthesis with a non-radioactive reverse transcriptase assay kit, according to the method described by the manufacturer (Boehringer-Mannheim).

To increase the expression level of RT, we constructed a derivative cell line of M153, which has the mouse amplification promoting sequence (muNTS1 element) (*33*) downstream of the M153-RT gene. This cell line, designated as M153E3, expresses mRNA for M153-RT in a 1.5- to 2-fold greater amount than the M153 cell line does.

Purification of RT—Wild-type and mutant RTs expressed in *E. coli* cells (QE70RT and QE70RTM153) were partially purified according to Gerard *et al.* (*34*). For further purification, the partially purified RT fraction was applied to an affinity column of Ni^{2+}-NTA bound agarose gel (Qiagen). The column was washed with buffer A, comprising 20 mM Tris-HCl (pH 8.0), 80 mM NaCl, 0.1% NP40, and 10% glycerol, and the RT protein was eluted with a 0-0.5 M linear gradient of imidazole in buffer A. The protein fraction eluted with 0.15-0.2 M imidazole, which was shown to consist mainly of the RT on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), was pooled, dialyzed against a Tris buffer comprising 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 100 mM NaCl, 1 mM DTT, 0.01% NP40, and 50% glycerol, and then used as the highly purified RT fraction in the experiments of Fig. 4 and Table IV. The protein concentration of the RT fraction was determined using a protein assay kit (Bio-Rad).

Reverse Transcriptase Assays—RT activity in the highly purified RT fractions (QE70RT as the wild-type RT and QE70RTM153 as a mutant RT) was assayed according to Verma *et al.* (*35*) with a slight modification. The reaction mixture (0.1 ml) comprising 25 mM Tris-HCl (pH 8.3), 10 mM DTT, 0.5 mM $MnCl_2$, 25 μg/ml actinomycin D, 0.1% NP40, 10 μg/ml poly(rC)/p$(dG)_{12-18}$ (Pharmacia), 8 μM dGTP including 370 kBq/ml [8-^{3}H]dGTP (Amersham), and highly purified RT, was incubated for 20 min at 37°C. Then, the synthesized poly(dG) was measured according to Goff *et al.* (*36*).

The competition of mutant RT with the wild-type RT in *in vitro* poly(dG) synthesis was monitored in the RT assay system described above, except that 250 ng/ml of poly(rC)/p$(dG)_{12-18}$ was used.

Binding of RT to Radioactive Poly(rA)-Oligo(dT)—Binding of RT to poly(rA) was monitored according to Lowe *et al.* (*20*) with the following modification. Purified RT was incubated at 37°C for 15 min in the reaction mixture for the RT assay as described above, except that ^{3}H-poly(rA) (Amersham) and oligo dT_{12-18} were used instead of poly(rC)/p$(dG)_{12-18}$. Then, the mixture was layered on a wet nitrocellulose filter disk (Millipore, HA 0.45 μm), and washed with 10 ml of ice-cold 50 mM Tris-HCl (pH 7.5) and 5 mM $MgCl_2$. The disks were air-dried and radioactivity was counted in a liquid scintillant. Commercial RNase-free bovine serum albumin (Pharmacia) was used in a blank reaction.

RESULTS

Interference with Mo-MuLV Proliferation by Mutant RT—The ability of RT mutants to interfere with proliferation of the wild-type virus was first assessed in the transient assay system, in which NIH3T3 cells were co-transfected with infectious proviral DNA (pMLV48) and either the wild type (pSM51) or mutant (pSM51-M153) RT gene-containing plasmid DNA. At the beginning of the experiments, we checked the condition for transfection of these plasmids and found that when pMLV48 alone was electroporated into NIH3T3 cells, release of the progeny viruses was detected at 5 days after transfection and the amount was maximum at 9 days after transfection (data not shown). Furthermore, we confirmed that the amount of viruses produced by cells transfected with pMLV48 alone was almost the same as that by cells co-transfected with pMLV48 and pSM51, indicating that the ability of progeny production by cells transfected with pMLV48 is not affected by co-transfection of pSM51. Using these conditions, pMLV48 and pSM51-M153 were co-transfected. As shown in Table I, the amount of the progeny produced by pMLV48 was much less than that in the cells co-transfected with pMLV48 and the wild-type RT plasmid, pSM51. The inhibition by the mutant plasmid at 7 and 9 days after transfection was 70 and 50%, respectively. In addition, the wild-type or mutant plasmid alone produced a negligible amount of progeny viruses. These results suggest that mutant RT acts as an active inhibitor of the generation of infectious virus particles by the proviral DNA.

To confirm the results of the above transient infection experiments, we constructed NIH3T3 cells which constitutively express mutant RT genes, as described in the text. Using these cells, we checked the expression of RT protein and the ability to inhibit Mo-MuLV proliferation. As to the expression of RT protein in these cells, we could not detect the RT protein by Coomassie-Blue staining on SDS-PAGE (data not shown). However, the existence of

the RT mRNA in these cells was detected by RT-PCR analysis using two primers covering the coding sequence of RT (Fig. 2). Thus, we tested whether or not these cells inhibit progeny virus formation upon Mo-MuLV infection using the XC cell method (*32*) and RT activity method in the culture medium of virus-infected cells. As shown in Table II, it was found that infectious progeny formation by the mutant RT-expressing cells was significantly reduced as compared to that by the wild-type RT-expressing cells, when these cells were infected with an equal amount of Mo-MuLV. In particular, proliferation of the virus at 7 days after infection was inhibited by an average of 90% in the cells expressing M153 RT. The above result was supported by the experiment monitoring RT activity in the culture medium of virus-infected cells, in which only 10% RT activity was found as compared to that of the wild-type RT cells (Table II, Column 4).

Figure 3 shows the time course of proliferation of Mo-MuLV in cells expressing either wild-type RT or M153 RT. Release of the virus from the wild-type RT-expressing cells increased up to 7 to 8 days after infection and then decreased gradually with prolonged infection time, whereas the mutant RT cells released a small amount of the virus in a similar manner to that of the wild-type RT cells. But the released virus number remained at about 20% of the wild-type cell level until 22 days after infection. These results suggest that this inhibition is not due to arrest of the adsorption step of the virus infection, but rather to suppression of the viral replication step itself.

Furthermore, we checked the effect on the inhibition rate of raising the level of RT expression inside the cells. However, a new construct, M153E3 cells, which express more mRNA for the mutant RT than M153 cells, did not show an elevated inhibition rate (Table III).

Binding of Mutant RT to Poly(rA) and Poly(dG) Synthesis—To cast light on the mechanism underlying the inhibi-

TABLE I. **Effects of mutant RTs on production of infectious progeny by Mo-MuLV proviral DNA in the transient assay system.** Infectious progeny production was monitored by the method of syncytium formation on the XC cells (*32*) as described in the text. +W represents the mixture of pMLV48 (10 μg DNA) and pSM51 (20 μg DNA). +M represents the mixture of pMLV48 (10 μg DNA) and M153 (20 μg DNA). W and M represent pSM51 (20 μg DNA) and pSM51-M153 (20 μg DNA) alone were used for transfection, respectively. Data represent the averaged results from two separate experiments.

Days after transfection	No. of syncytia/0.1 ml sup			
	+W	+M	W	M
4	14	14	0	0
5	38	17	4	0
6	20	16	4	0
7	237	72	0	0
9	918	448	4	0

TABLE II. **Inhibition of Mo-MuLV proliferation in cells expressing mutant RTs at 7 days after infection.** Various cell lines which express the wild-type RT (WT) or mutant RTs were infected with an equal amount of Mo-MuLV. After adsorption, cells were washed, placed in fresh medium and then incubated at 37°C. Released viruses were monitored as described in the text. No. of syncyt. represents the average number of syncytia in 0.1 ml of supernatant in 3 separate experiments. *One unit corresponds to 0.2 ng of RT.

Cell line	No. of syncyt.	Inhibition (%)	RT activity (unit*)
WT	1,600	—	0.2
N17	597	63	
M151	450	72	
M153	162	90	0.02
M134	645	60	

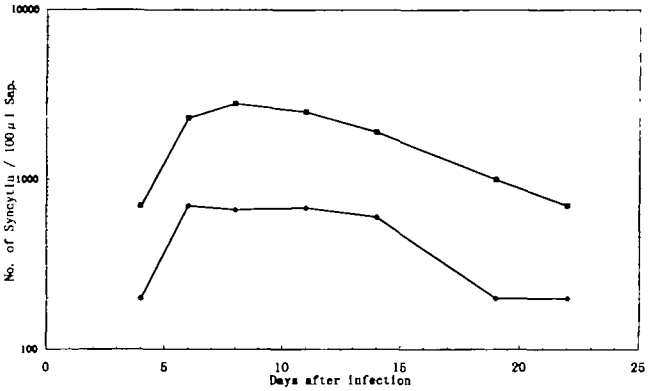


Fig. 3. **Time course of proliferation of Mo-MuLV in cells expressing the wild type or M153 RT.** Experiments were performed in a similar manner to that described in Table II. ■: wild-type, and ◆: M153 RT.

TABLE III. **Comparison of inhibitory effects of M153 and a new mutant M153E.** No. of syncyt. represents the average number of syncytia formed in 0.1 ml of supernatant. The experiments were carried out as described in Table II. WT represents a cell line which contains the wild-type RT gene and the NTS1 sequence (*33*). *Amount of RT mRNA was estimated as radioactivity hybridized to RT gene (cpm/1 μg mRNA). Data presented are the averaged values in two separate experiments.

Cell line	No. of syncyt.	Inhibition (%)	Amount of mRNA*
WT	1,065	—	—
M153	155	86	100
M153E3	340	69	143

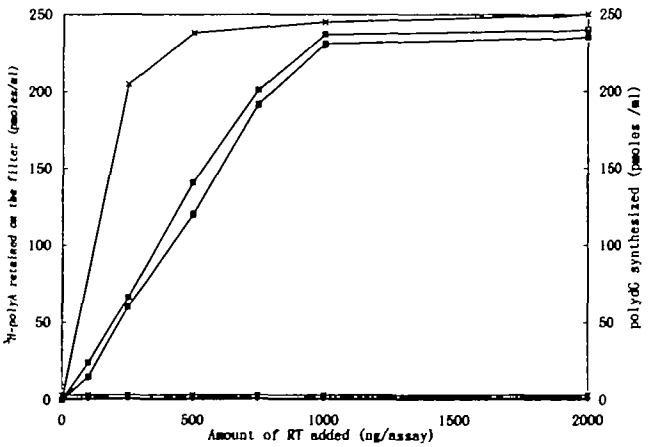


Fig. 4. **Poly(dG) synthesis by RT and binding of RT to poly(rA).** Poly(dG) synthesis was carried out in the same reaction mixture as described in the text. The binding of RT was performed in the above reaction mixture for poly(dG) synthesis except that poly(rA) and oligo(dT) were added instead of poly(rC)/p(dG)$_{12-18}$. −×−: wild-type (QE70RT); −*−: QE70RTM153 for poly(dG) synthesis. □: wild-type(QE70RT); ■: QE70RTM153 for binding of RT, and ◆: bovine serum albumin, respectively.

TABLE IV. **Inhibition of *in vitro* poly(dG) synthesis with wild-type RT by QE70RTM153 RT.**

Mutant RT[a] or BSA (ng)	Inhibition (%)[b] in the system having the following amounts of wild-type RT (ng)		
	10	100	1,000
100	11	ND	ND
500	45	12 (3)	ND
1,000	88	31 (2)	26
2,000	98	60 (4)	60
5,000	ND	91 (2)	87

[a]Represents QE70RTM153 as a mutant RT or bovine serum albumin (BSA) added to the wild-type RT (QE70RT)-dependent poly(dG)-synthesizing system. [b]Represents the average values in 2 separate experiments with duplicate assays, calculated by use of the following equation: 100 (%) minus the relative activity (%) calculated by dividing the amount of poly(dG) synthesized by the wild-type RT in the presence of various amounts of mutant RT with that of poly(dG) synthesized by the wild-type RT alone. Poly(dG) synthesis was carried out as described in the text using 250 ng/ml of poly(rC)/$p(dG)_{12-18}$ and 10, 100, or 1,000 ng/0.1 ml of the wild-type RT. The values in parenthesis represent the inhibition rate by BSA, instead of mutant RT, added to the reaction mixture containing 100 ng of the wild-type RT. ND represents not determined.

tion of viral proliferation by mutant RT, we examined whether a purified mutant RT can bind to the template RNA. In this connection, we also checked the poly(dG) synthesis using these RTs. The results (Fig. 4) clearly show that the mutant RT exhibited no polymerization activity even at the concentration of 1 μg/assay, whereas the wild-type RT synthesized poly(dG) dependently on the amount of the enzyme and its activity reached almost maximum at about 500 ng/assay in this system.

Concerning the binding activity of RT to template RNA, mutant RT was found to bind to the template RNA as effectively as the wild-type RT did, because the amount of poly(rA) retained on the nitrocellulose filter increased almost linearly and in a similar manner with increasing amounts of both RTs. It reached the maximum at about 2 μg/assay of RT. However, commercial RNase-free bovine serum albumin could not hold poly(rA) on the filter. We thus conclude that this mutant RT still retains the ability to bind to template RNA, despite the fact that it has lost the polymerization activity.

Inhibition of In Vitro cDNA Synthesis with Polymerization-Defective RT—Using the same RT preparations as used in Fig. 4, we examined whether mutant RT can inhibit *in vitro* poly(dG) synthesis by the wild-type RT. As shown in Table IV, when various amounts of mutant RT were added to the reaction mixture containing 10,100 and 1,000 ng/assay of the wild-type RT, the poly(dG) synthesis was distinctly reduced with increasing amount of mutant RT. Moreover, in this system, 2 to 5 μg of mutant RT gave nearly maximum inhibition of the poly(dG) synthesis. Since the template RNA present in this system was able to bind to about 2 μg of RT at the maximum level (see Fig. 4), these amounts of mutant RT seem to be needed to compete sufficiently with the wild-type RT. In the system containing 100 ng/assay of the wild-type RT, bovine serum albumin exhibited only slight inhibition of poly(dG) synthesis. In this connection, we confirmed that no RNase activity was found in 2-5 μg/assay of the mutant RT (data not shown). These results thus suggest that the mutant RT interferes trans-dominantly with the polymerization of substrates occurring with the wild-type RT.

DISCUSSION

In this study, we investigated the dominant-negative inhibition of Mo-MuLV proliferation by polymerization-defective mutants of RT. To assess the ability of RT mutants to inhibit viral replication, we constructed various plasmids harboring the RT gene in which a single amino acid in the conserved sequence was altered. Using these plasmids, we demonstrated that mouse cells harboring these plasmids were resistant to Mo-MuLV proliferation while the mouse cells harboring an isogenic plasmid with the wild-type RT gene were sensitive. In particular, the mutant cells having an amino acid substitution at the first aspartic acid residue of the conserved YXDD sequence were noteworthy (M151 and M153, Table II). Since we have detected the existence of mRNA for RT in these cells, it can be said that this resistance is dependent on the polymerization-defective RT gene. Although we could not detect the RT protein produced in these cells on SDS-PAGE, we think that this was a consequence of either a low level of production or instability of the RT protein, rather than no production. As to the level of RT expression necessary for substantial inhibition, our data suggest that a relatively low level of expression of the mutant allele is sufficient to repress virus replication. In addition, raising the level of the expression of RT mRNA did not increase the inhibition rate (Table III). This is consistent with Golemboski *et al.*'s report that a non-detectable amount of mutated viral polymerase could inhibit the replication of a plant virus (*13*).

At present, little is known about the precise mechanism underlying the inhibition of Mo-MuLV infection by defective RTs. However, it is remarkable that although mutant RTs had no polymerization activity, they bound to the template RNA as well as the wild-type RT did (Fig. 4). This is in good agreement with the results reported by Lowe *et al.* (*20*) and Inokuchi *et al.* (*25*). Furthermore, Table IV shows that a polymerization-defective mutant RT inhibited the *in vitro* poly(dG) synthesis by the wild-type RT, suggesting that the mutant RT competes with the wild-type RT for binding to the template RNA. Accordingly, it could be said that the pre-existing mutant RT competed with the wild-type RT derived from the invading viral particles and/or produced under the direction of the viral RNAs during the replication cycle, and then inactivated the subsequent function of the viral RNA, resulting in significant and long-term suppression of the viral replication. Furthermore, it is also possible that the mutant RTs which had been assembled into progeny particles inhibit the second and following rounds of the viral replication in the same manner.

The results presented here demonstrate that mouse cells harboring the RT gene mutated at the YXDD sequence, which was shown to be important for polymerization activity, acquire immunity against infection by the virus from which the RT gene was derived. As already reported for bacteriophages (*3*) and plant viruses (*13-15*), this defective RT-mediated inhibition system is also applicable to animal viruses including retroviruses, which have the YXDD sequence in the polymerase.

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