JOURNAL OF VIROLOGY, Dec. 1975, p. 1476-1482
Vol. 16, No. 6

Printed in U.S.A.

Thermolabile Reverse Transcriptase of a Mammalian Leukemia Virus Mutant Temperature Sensitive in Its Replication and Sarcoma Virus Helper Functions

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Received for publication 12 June 1975

Three temperature-sensitive mutants of the Rauscher strain of murine leukemia virus are defective in early post-penetration functions required both for leukemia virus infection and for initiation of transformation of cells by their pseudotypes of murine sarcoma virus. In the present study, the reverse transcriptase of one of these mutants (ts 29) is shown to be thermolabile compared with the enzymes of the wild-type virus and several other temperature-sensitive mutants. These findings provide evidence that the reverse transcriptase is required both for leukemia virus infection and for initiation of transformation by the replication-defective murine sarcoma virus genome.

Conditional lethal mutants of avian (6, 13, 27, 36) and mammalian (21, 24, 35) type C RNA viruses have been isolated in order to analyze the mechanisms of viral replication and to provide markers for mapping the viral genome. With mammalian type C viruses, temperature-sensitive (ts) mutants of the Kirsten (24), Rauscher (21), and Moloney (35) strains of murine leukemia virus (MuLV) have been isolated. Several ts mutants of Rauscher MuLV (R-MuLV), defective in post-penetration functions, have been characterized with respect to the stages at which viral replication is blocked at the nonpermissive temperature (39 C) (21, 23, 25). Viruses designated as early mutants do not synthesize viral antigens, and murine sarcoma virus (MSV) pseudotypes of these mutants do not transform cells at 39 C. In contrast, viruses designated as late mutants synthesize wild-type (wt) levels of viral antigens, and MSV pseudotypes of these viruses transform cells as efficiently as wt at 39 C.

A MuLV gene product likely to be required at an early stage of infection is the RNA-dependent DNA polymerase (reverse transcriptase). Previous studies with Rous sarcoma virus have shown that two ts mutants defective in early functions possess thermolabile DNA polymerases (16, 17, 33). To examine the possibility that one of the R-MuLV ts mutants might possess a thermolabile reverse transcriptase, the heat inactivation kinetics of the DNA polymerases of several R-MuLV ts mutants have been compared with that of the wt viral enzyme. The results of the present study indicate that the reverse transcriptase is a mammalian type C virus gene product required for leukemia virus infection and for transformation of cells by sarcoma viruses.

MATERIALS AND METHODS

Viruses. The isolation of ts mutants of R-MuLV and preparation of pseudotypes using the Kirsten strain of MSV has been described (21, 23). Wild-type and mutant viruses were grown in NIH/3T3 cells (12) at 31 C. The mutant viruses were checked for reversion to wild-type by testing their ability to grow at the nonpermissive temperature, using the XC plaque assay (14).

Reverse transcriptase assays. The reaction mixture for determining reverse transcriptase activity was the following: 50 mM Tris-hydrochloride, pH 7.8; 90 mM KCl; 2 mM dithiothreitol (DTT); 0.1 mM $MnCl_2$; 0.5% Triton X-100 (vol/vol); 500 ng of poly(rA) (collaborative Research, Waltham, Mass.); 375 ng of oligo(dT)$_{(12-18)}$ (Collaborative Research); and 5.9 μM [*methyl*-^{3}H]TTP (7.55 $\times$ 10^3 counts/min per pmol) (Schwarz/Mann, Orangeburg, N.Y.) in a final volume of 0.1 ml. Assays were carried out in siliconized glass tubes for 60 min at 34 C, and samples were processed for measurement of radioactive product as previously described (28).

Purification of reverse transcriptase. The procedure of Ross et al. (19) was followed with the exception that the DEAE-dextran step was omitted. Viruses were purified by isopycnic centrifugation in a linear sucrose gradient (20 to 60%, wt/vol), using a Beckman CF32 rotor. The virions (1 to 2 mg of protein) were disrupted by treatment with 1.0% Triton X-100 in the presence of 0.5 M KCl, 1 mM DTT, and 20% glycerol for 1 h at 4 C. The mixture was chromatographed on a Sephadex G-100 column (2.5 by 100 cm) that was equilibrated and eluted with 25

mM Tris-hydrochloride, pH 7.8, 1 mM DTT, 0.3 M KCl, 0.01% Triton X-100, and 20% (vol/vol) glycerol. The peak of polymerase activity was concentrated approximately fivefold by Amicon (Lexington, Mass.) membrane ultrafiltration and then dialyzed against 50 mM imidazole-hydrochloride, pH 6.5; 1 mM DTT; 0.01% Triton X-100; 20% glycerol (buffer A); and 0.1 M KCl. The enzyme solution was then applied to a column (1 by 20 cm) of Whatman phosphocellulose (P-1) (Reeve Angel, Clifton, N.J.), equilibrated with buffer A, and eluted by using a linear gradient of 0.2 to 0.5 M KCl in buffer A. Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) (1 mg/ml) was added to the polymerase solutions to prevent absorption of the enzymes to glassware. Enzyme solutions were stored at −190 C.

Heat inactivation kinetics. For determining the kinetics of heat inactivation of reverse transcriptase activity of virion-associated or purified enzymes, the polymerase preparations were incubated with 100 mM Tris-hydrochloride, pH 7.8, 180 mM KCl, 4 mM DTT, 0.2 mM $MnCl_2$, and 0.1% Triton X-100, in a total volume of 1.0 ml (mixture I). After heating for various time periods (see figure legends), 0.05-ml samples were withdrawn and added to 0.05 ml of a solution containing radioactive substrate and template-primer (mixture II). This mixture was kept on ice for not more than 60 min and then assayed for activity after incubation at 34 C for 60 min. The synthesis of radioactive product was linear with respect to time. The amounts of templates and primers used for these experiments in mixture I were as follows: poly(rA), 500 ng, and oligo(dT), 375 ng; poly(rA), 500 ng, and poly(dT), 200 ng; poly(rC)·oligo$(dG)_{(12-18)}$ (Collaborative Research), 100 ng. Mixture II also contained either 0.59 nmol of [*methyl*-^{3}H]TTP or 1.4 nmol of [8-^{3}H]dGTP (3.1×10^3 counts/min per pmol) (Schwarz/Mann). The optimum $MnCl_2$ concentration for assays with poly(rC)·oligo(dG) was 1 mM (unpublished observations); therefore, $MnCl_2$ (90 μmol) was also included in mixture II. In some experiments, the enzymes were heated in mixture I in the presence of templates. Thus, amounts of templates and primers used per 0.05 ml of mixture I were twice those listed above. In all experiments, amounts of enzymes were used such that 10^5 counts/min was detected in the controls.

RESULTS

Thermal inactivation of reverse transcriptase activity of disrupted virions. The physiological properties of the ts mutants of R-MuLV investigated here are summarized in Table 1. To test the thermolability of the reverse transcriptase activity of each mutant, virions were disrupted with Triton X-100 and incubated at 40 C, the nonpermissive temperature for virus replication; surviving enzyme activity was then assayed at 34 C. In these experiments, virus-containing culture fluids were used as the source of enzyme. The results (Fig. 1A) indicate that the reverse transcriptase activity of one early mutant, ts 29, was more thermolabile than that of the wt enzyme. The time required for inactivation of one-half the ts 29 enzyme

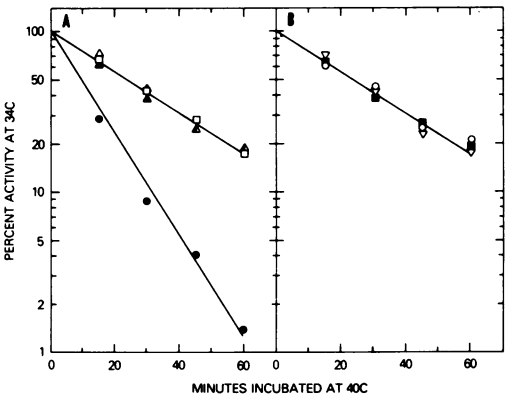

FIG. 1. *Thermal inactivation of reverse transcriptase activity of disrupted virions. Viruses were incubated at 40 C and assayed at 34 C, using poly(rA)·oligo(dT) as described in the text. (A) ts 29, ●; ts 17, □; ts 19, △; wild type, ▲. (B) ts 18, ▽; ts 25, ■; ts 26, ○.*

TABLE 1. *Properties of temperature-sensitive mutants of R-MuLV*

Virus strain	Function completed at the nonpermissive temp[a]			
	Absorption penetration	Viral antigen synthesis	Transformation of cells by MSV pseudotype	Particle synthesis
Wild-type R-MuLV	+	+	+	+
Early mutants				
ts 17	+	−	−	−
ts 19	+	−	−	−
ts 29	+	−	−	−
Late mutants				
ts 18	+	+	+	−
ts 25	+	+	+	−
ts 26	+	+	+	−

[a] These data are based on previously published work (21, 23–25).

activity ($t_{1/2}$) was 9 min in contrast to 24 min for the wt enzyme. The $t_{1/2}$ values for the enzymes of the other early mutants (ts 17 and ts 19) (Fig. 1A) and each of three late mutants (ts 18, 25, and 26) were indistinguishable from that of the wt virus (Fig. 1B).

Thermolability of enzymes purified from ts 29 and wt virions. To show that the greater thermolability of the reverse transcriptase activity of ts 29 was due to a defect in the enzyme rather than an indirect effect of disrupted virion preparations, the reverse transcriptases of ts 29 and wt viruses were partially purified. The purification procedures resulted in 72 and 69% recovery of the activity of the wt and ts 29 enzymes, respectively (Table 2). The protein concentrations in the enzyme preparations were very low (<5 μg/ml), so that assessment of the final degree of purity was not possible. However, virion p30 and p12 polypeptides were not detected in these preparations by sensitive radioimmunoassay procedures (p12 < 0.2 ng/ml, p30 < 2 ng/ml) (22, 29). Since the respective concentrations of p30 and p12 in the starting virion preparations were approximately 300 and 90 μg/ml, the purification procedures resulted in at least 1.5×10^5- and 4.5×10^5-fold reductions in the concentrations of these virion structural polypeptides.

During purification, no significant differences were detected with respect to the stability or chromatographic behavior of the enzymes. Both mutant and wt polymerases eluted from Sephadex G-100 columns at a position corresponding to approximately 70,000 daltons and eluted from phosphocellulose columns at 0.3 M KCl as previously reported for the R-MuLV DNA polymerase (19). The template specificity and antigenic characteristics of the partially purified enzymes were also examined. Each enzyme responded similarly to three synthetic template-primer combinations [poly(rA)·oligo(dT) > poly(rA)·poly(dT) > poly(rC)·oligo(dG)] (Table 3). The poly(rA)·oligo(dT)-supported activity of both enzymes was inhibited by 98%, using a 1:20 dilution of an antiserum prepared against the purified DNA polymerase of R-MuLV (28).

The thermal inactivation kinetics of the purified enzymes were next examined. The heat lability of the wt and ts 29 enzyme preparations were compared over a range of temperatures (40 to 50 C). Although the ts 29 polymerase was more labile than the wt at each temperature tested, the greatest differences were observed at 45 C. Thus, the enzyme preparations were heated at 45 C and then assayed for surviving enzyme activity at 34 C (Fig. 2), using the synthetic template poly(rA)·oligo(dT). The activity of the ts 29 enzyme decayed twice as fast as that of the wt ($t_{1/2}$, ts 29 = 6 min; $t_{1/2}$, wt = 13 min) (Fig. 2). The thermolability of the reverse transcriptase purified from a second clone of wt virus was also examined. The $t_{1/2}$ values for both wt clones were indistinguishable. To exclude the possibility that the greater thermolability of ts 29 polymerase may have been due to an inhibitor in the preparation, ts 29 and wt reverse transcriptases were mixed and heated at 45 C. At each time interval, the activity of the mixture was the same as the sum of the activities of both enzymes heated alone (Table 4).

Effect of synthetic templates on the thermolability of purified viral enzymes. Mammalian type C viral RNA-dependent DNA polymerases synthesize polydeoxyribonucleotides using several different synthetic ribohomopolymer templates (in the presence of the appropriate primers) (4), as well as with natural RNA at a much lower efficiency (34). It was of interest, therefore, to determine the thermolability of the ts 29 enzyme by using other template-primer combinations. Of the templates tested, only poly(rA)·poly(dT) and poly(rC)·oligo(dG) supported sufficient activity with the levels of enzyme obtainable (Table 3). The $t_{1/2}$ values for thermal inactivation of ts 29 enzyme were 5 min with poly(rA)·oligo(dT), 6.5 min with poly(rA)·poly(dT), and 5 min with poly(rC)·oligo(dG) (Fig. 3A–C). In contrast, the $t_{\frac{1}{2}}$ values for wt enzyme were 13, 17, and 21 min with the respective templates.

TABLE 2. *Purification of reverse transcriptase from wild-type and ts 29 virions*

Purification step	Wild-type		ts 29	
	Total enzyme activity[a]	Recovery (%)	Total enzyme activity[a]	Recovery (%)
Purified virions	3.7×10^5	100	1.6×10^5	100
Sephadex G-100	6.3×10^5	169	2.6×10^5	162
Phosphocellulose	2.2×10^5	59.3	1.1×10^5	69.0

[a] Picomoles of [^{3}H]TMP incorporated per hour per total volume of enzyme preparation.

TABLE 3. *Template specificity of partially purified enzymes*[a]

Enzyme	Activity with:			Ratio of activities	
	Poly(rA)·oligo(dT)	Poly(rA)·poly(dT)	Poly(rC)·oligo(dG)	Poly(rA)·poly(dT)/ poly(rA)·oligo(dT)	Poly(rC)·oligo(dG)/ poly(rA)·oligo(dT)
wt	7,350	2,670	1,440	0.36	0.20
ts 29	2,720	1,240	620	0.46	0.23

[a] Picomoles of [*methyl*-^{3}H]TMP or [8-^{3}H]dGMP incorporated per milliliter of enzyme per hour at 34 C. The reaction mixtures for determination of activity using poly(rA)·oligo(dT) and poly(rA)·poly(dT) were exactly as described in the text. The concentrations of poly(rA) and poly(dT) were 5 and 1 μg/ml, respectively. In assays using poly(rC)·oligo(dG), the template-primer concentration was 2 μg/ml, the $MnCl_2$ concentration was 1 mM, and the [8-^{3}H]dGTP concentration was 14 μM.

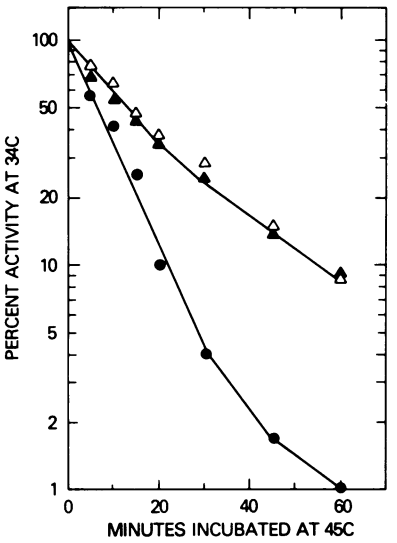

FIG. 2. *Thermal inactivation of partially purified reverse transcriptases. Enzymes from ts 29 (●) and two separate clones of wild-type virus (clone 1, ▲; clone 2, △) were incubated at 45 C and assayed at 34 C, using poly(rA)·oligo(dT) as described in the text.*

The effect of incubating the enzymes at 45 C in the presence of templates was also examined. In the presence of template, the half-lives of both ts 29 and wt polymerase activities were increased (Fig. 3D–F). For example, when the enzymes were heated in the presence of poly(rA)·poly(dT) (Fig. 3E), the $t_{1/2}$ values for ts 29 and wt activities were increased 4.8- and 4.4-fold, respectively. However, the differences between the $t_{1/2}$ values of the ts 29 and wt enzymes remained similar to those observed in the absence of template.

Heat inactivation kinetics of purified reverse transcriptases of other early mutants. The results above demonstrate that an early mutant, ts 29, possesses a ts reverse transcriptase. Two other mutants, ts 17 and ts 19, were similar to ts 29 in that they did not synthesize viral antigens and their pseudotypes of MSV did not initiate transformation at 39 C (Table 1). The reverse transcriptases of these mutants were purified by the procedures described in Materials and Methods, and their thermolabilities were compared with that of the purified wt polymerase (Fig. 4). The $t_{1/2}$ values for thermal inactivation of the wt, ts 17, and ts 19 enzymes, assayed by using three different template-primer complexes, were nearly indistinguishable. The values were 15 min with poly(rA)·oligo(dT), 18 min with poly(rA)·poly(dT), and 21 min with poly(rC)·oligo(dG).

DISCUSSION

The present studies demonstrate that an early mutant of R-MuLV, ts 29, possesses a thermolabile reverse transcriptase. The thermolability of the ts 29 polymerase was observed both in disrupted virions and in purified enzyme preparations. These findings suggest that the reverse transcriptase is required for leukemia virus infection and is coded for by the mammalian type C viral genome.

Most isolates of mammalian sarcoma viruses have been shown to code for functions involved in expression of the transformed state but to lack the ability to replicate as infectious viruses (1–3, 5, 9, 11, 20). Previous studies have shown that leukemia viruses provide their envelope to the defective mammalian sarcoma virus (10), thus enabling it to initiate a new cycle of infection and transformation. The present findings have shown that an early leukemia virus mutant, which fails to act as a helper for Kirsten MSV at the nonpermissive temperature, possesses a thermolabile reverse transcriptase. Thus, the reverse transcriptase appears to be a post-penetration leukemia viral helper function

TABLE 4. *Thermal inactivation of a mixture of reverse transcriptases purified from wild-type and ts 29 virions*

Time at 45 C[b]	Enzyme activity remaining at 34 C[a]			
	wt heated separately	ts 29 heated separately	Sum of activities of enzymes heated separately	Mixture of wt and ts 29
0	21.4	16.3	37.7	37.8
5	16.7	10.7	27.4	27.1
10	13.6	8.6	22.2	23.3
15	12.0	5.5	17.5	17.2
30	7.9	0.9	8.8	7.1
45	4.0	0.2	4.2	4.1
60	3.2	0.1	3.2	2.4

[a] Picomoles of [^{3}H]TMP incorporated into acid-insoluble material per hour per 50 μl of heating mixture.
[b] Enzymes were heated and assayed by using the conditions described in Fig. 2.

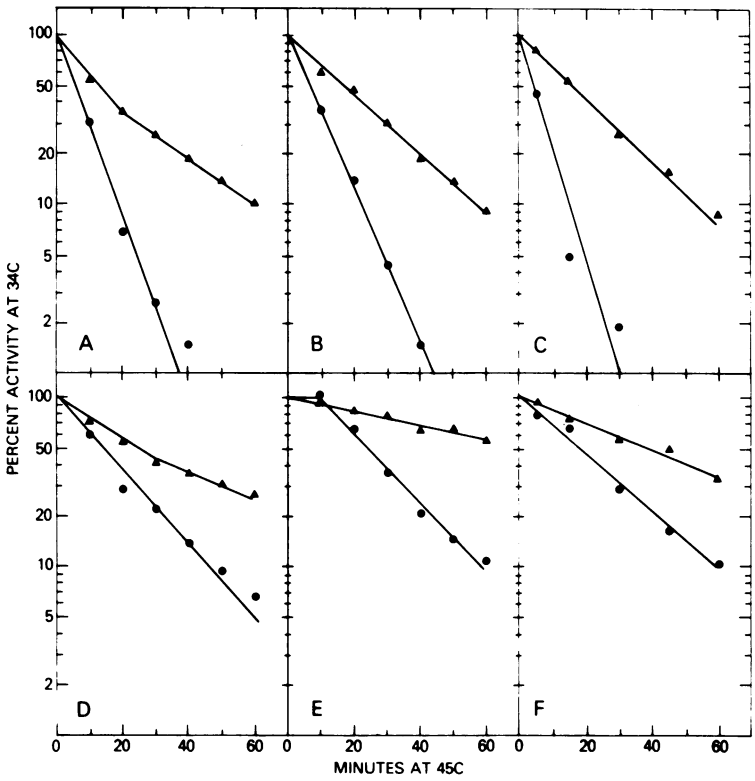

FIG. 3. *Thermal inactivation of partially purified reverse transcriptases in the presence and absence of synthetic templates. Wild type (▲) and ts 29 (●) enzymes were incubated at 45 C in the absence of synthetic templates and then assayed with (A) poly(rA)·oligo(dT); (B) poly(rA)·poly(dT); or (C) poly(rC)·oligo(dG). Enzymes were also incubated at 45 C in the presence of (D) poly(rA)·oligo (dT); (E) poly(rA)·poly(dT); or (F) poly(rC)·oligo(dG), and then assayed at 34 C.*

necessary for initiation of transformation by the defective sarcoma viral genome.

The isolation of a spontaneous revertant of ts 29 to wt would help to confirm that the polymerase defect was responsible for the early block to replication. However, numerous attempts to obtain such revertants have been unsuccessful. These findings suggested the possibility that this virus contains more than one mutation. In fact, recent studies performed in collaboration with V. Kalnins, University of Toronto, indicate that ts 29 also possesses a late ts replication defect associated with impaired virion maturation at the stage of budding from the cell

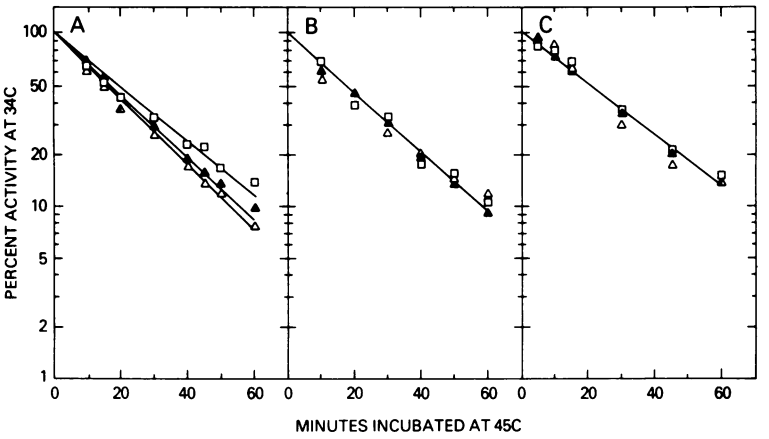

FIG. 4. *Thermal inactivation of partially purified polymerases isolated from other early mutants. The conditions used were the same as described in Fig. 3, except that templates were omitted from the heating mixture. Symbols: ▲, Wild type; □, ts 17; △, ts 19. Enzymes were incubated at 45 C and assayed at 34 C with (A) poly(rA)·oligo(dT); (B) poly(rA)·poly(dT); and (C) poly(rC)·oligo(dG).*

membrane (unpublished data). It would seem unlikely that the late defect in virus budding would be due to the ts polymerase. However, the possibility that the ts polymerase results from a neutral mutation and that some other defect is responsible for the early ts block to virus infection can not be rigorously excluded in the absence of a revertant.

The thermolabilities of the reverse transcriptase activities in disrupted virions and in purified enzyme preparations of two other early mutants (ts 17 and ts 19) were found to be indistinguishable from that of the wt enzyme. These findings suggest that early leukemia viral gene functions in addition to the reverse transcriptase may be required for integration of the type C viral genome in newly infected cells. Alternatively, the ts 17 and ts 19 polymerases may be defective in reverse transcriptase activities other than those studied here. It is possible that the enzymes of these mutants may lack the ability to copy viral RNA or to degrade RNA-DNA hybrids (7, 18, 31, 32) at the restrictive temperature.

Two ts mutants of Rous sarcoma virus, defective in early functions necessary for the initiation of both replication and cell transformation, have previously been shown to possess thermolabile DNA polymerases (16, 17, 33). The ts lesion in these avian mutant enzymes is thought to be due to defective template binding, since template protected wt but not mutant enzymes from thermal inactivation (33). This is in contrast to the present findings in which ts 29 and wt polymerases were each stabilized by template to the same extent.

Recent work with avian and murine type C viruses has begun to elucidate the early intracellular steps involved in the synthesis of viral DNA and its subsequent integration into the host cell genome. Thus, covalent viral RNA-DNA hybrids (15, 26) and closed circular viral DNA duplexes (8, 30) have been demonstrated in the cytoplasm of newly infected cells. Subsequently, the viral DNA becomes integrated into the genome of the cell (30). The availability of ts mutants of MuLV defective in early replication functions should aid in determining which of these steps are catalyzed or regulated by viral gene products.

ACKNOWLEDGMENTS

We thank David Baltimore for helpful advice and Marjorie M. Golub for excellent technical assistance.

This work was supported in part by Public Health Service contract no. NCI-E-73-3212 of the Virus Cancer Program of the National Cancer Institute.

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