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Mechanism of Carcinogenesis by RNA Tumor Viruses, I. An RNA-Dependent DNA Polymerase in Murine Sarcoma Viruses*

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Abstract. A highly active and stable DNA polymerase was found in purified preparations of two murine sarcoma viruses. Enzyme activity is not detected in most virus preparations unless they are treated with low concentrations of a nonionic detergent such as Nonidet P-40. The incorporation of labeled thymidine triphosphate requires all four deoxyribonucleoside triphosphates and either Mg^{2+} or Mn^{2+}. Enzyme activity is proportional to virus concentration and is linear with time up to 90 min. That the template is RNA is suggested by the reduction in polymerase activity upon treatment of murine sarcoma virus with RNase, and by the absence of detectable amounts of DNA in the virus. That the product is DNA is shown by the incorporation of all four deoxyribonucleoside triphosphates into an acid-insoluble product which is stable in alkali, is destroyed by DNase, sediments in alkaline sucrose gradients with a sedimentation coefficient of 7 S, and bands in isopycnic CsCl gradients with a mean buoyant density of 1.700.

The RNA-containing leukemia and sarcoma viruses possess two unique properties which distinguish them from other RNA-containing animal viruses and bacteriophages:[1] (1) DNA synthesis is required early during infection for virus replication and for cell transformation, i.e., if DNA synthesis is inhibited during the first 8–12 hr of infection viral replication and cell transformation are irreversibly aborted;[2] this requirement can be fulfilled in the presence of cycloheximide, an inhibitor of protein synthesis. (2) Actinomycin D, an inhibitor of DNA dependent RNA transcription, blocks virus replication at all times after infection, suggesting that functioning DNA is required continuously for the synthesis of viral progeny.[2,3]

In a recent review,[1] we concluded on the basis of the above inhibition studies and other recent experiments that only two mechanisms are consistent with all the data: (1) the virus particle (virion) contains an enzyme which copies DNA from viral RNA, or (2) cellular DNA binds viral RNA, perhaps stabilizing the viral genome. Based mainly on inhibitor studies with Rous sarcoma virus, Temin[2] has proposed that DNA that contains viral information is synthesized early after infection, presumably by a reversal of genetic transcription, i.e., viral RNA → DNA, and that newly synthesized viral DNA is a template for the synthesis of viral RNA.

We report here evidence for mechanism (1): purified preparations of two murine sarcoma viruses (MSV), the Harvey isolate, MSV(H), and the Moloney isolate, MSV(M), contain a highly active and stable DNA polymerase that incorporates all four deoxyribonucleoside triphosphates into DNA. The properties of the DNA polymerase, the nature of the template, and the properties of the DNA product are described. The possible role of transcribed viral DNA in virus replication and in cell transformation is discussed in this paper. Very recently, DNA polymerase activity was detected in Rous sarcoma virus by Temin and Mizutani,[4] in Rauscher murine leukemia virus by Baltimore,[4] and, more recently, in feline leukemia virus by Spiegelman.[4]

Materials and Method. Materials: The following items were purchased: dATP, dGTP, dCTP, and dTTP from the Sigma Chemical Co., St. Louis; [^{3}H]dTTP from the New England Nuclear Corp. and from Schwarz Biochemicals; α-^{32}P-labeled dATP, dGTP, dCTP, and dTTP from the International Chemical and Nuclear Corp.; Nonidet P-40 from the Shell Chemical Co.; polyethylene glycol (avg mol wt 6,000–7,500) from Matheson, Coleman and Bell, Inc.

Virus and cells: A seed culture of MSV(H), twice focus purified, was kindly provided by Dr. H. Temin. We transformed mouse embryo cultures (NIH-Swiss) with MSV(H), deriving the MEH cell line, which grows in suspension after 60 passages in monolayer in Eagle's MEM medium[5] supplemented with 10% tryptose phosphate broth and 10% fetal calf serum. The MSV(M) transformed rat cell line, 78A1, kindly provided by Drs. M. Benyesh-Melnick and N. Biswal, was grown as monolayers in Eagle's MEM medium supplemented with 10% fetal calf serum. Media harvested from MEH and 78A1 cells were frozen at −35°C until used for purification of virus.

Virus purification: Virus was isolated and purified from MEH or 78A1 growth media by the procedure of Duesberg and Robinson[6] with a slight modification. Six liters of cell media were stirred for 1 1/2 hr at 4°C after the addition of polyethylene glycol (10%) and sodium chloride (0.5 M). The virus-containing precipitate was centrifuged at 2700 × g for 20 min, resuspended in buffer, and further purified as described.[6]

DNA polymerase assay: The procedures described previously[7] were used with slight modifications. The incubation mixture (100 μl) contained 40 mM Tris, pH 8.1; 5 mM dithiothreitol; 2.5 or 5 mM $MgCl_2$; 0.1 mM dATP, dGTP, dCTP; 10 μCi of [^{3}H]dTTP (4.3–11.4 Ci/mmol) and 2–20 μg of viral protein. NaCl (30 mM) was included in the assay but does not appear to be necessary for enzyme activity. Purified MSV was dialyzed against 500 volumes of 0.01 M Tris, pH 8.1 for 3 hr before assay for polymerase. Nonidet P-40 (0.01%) was added to the virus at the time of assay.

After incubation at 37°C for various times (usually 1 hr), the reaction was stopped by the addition of 150 μl of cold 1 N perchloric acid (PCA) and 100 μl of carrier calf-thymus DNA (1 mg/ml). Radioactive DNA was isolated as described previously,[7] and counted in a scintillation spectrometer.

Results. Incorporation of [^{3}H]dTTP into an acid-insoluble form by purified MSV (DNA polymerase activity of MSV): All ten preparations of purified MSV(H) that we tested for DNA polymerase activity were able to incorporate [^{3}H]dTTP into an acid-insoluble form upon treatment with 0.01% Nonidet P-40 (NP-40). Other nonionic detergents, including Triton X-100, Tween 40, and Tween 80, also were effective in unmasking DNA polymerase activity (Table 1). One MSV(H) preparation incorporated [^{3}H]dTTP without added detergent but the addition of 0.01–0.025% NP-40 increased incorporation to 3000–3500 cpm (Fig. 1). Electron micrographs of detergent-treated virion after the polymerase reaction revealed strand-containing structures, probably cores with viral RNA.

MSV(H) and MSV(M) have very active DNA polymerases which are activated to different extents by NP-40 (Table 2). The DNA polymerase of purified virus is stable. No activity of dialyzed virus was lost after storage at 4°C for two weeks, and not more than 50% loss occurred after 2 months.

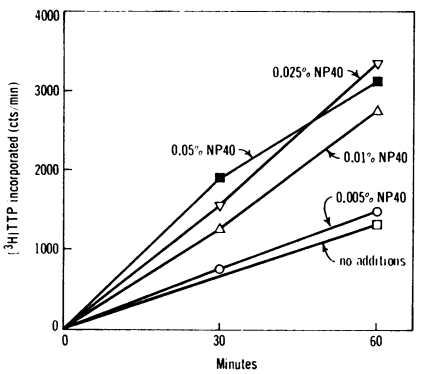


FIG. 1.—Effect of different concentrations of Nonidet P-40 on the incorporation of dTTP by the MSV DNA polymerase. The standard assay was used with 10 μCi of [3H]dTTP (11.4 Ci/mmol) and 10 μg of viral protein.

Requirements for the DNA polymerase reaction: The conditions for optimal incorporation of [3H]-dTTP into an acid-insoluble product were determined using NP-40 to activate enzyme activity. Polymerase activity requires all four deoxyribonucleoside triphosphates and a divalent cation, is proportional to virus concentration, and is linear for at least 90 min.

TABLE 1. *Effect of detergents on MSV DNA polymerase activity.*

Treatment	Incorporation of [3H]dTTP (cpm)
None	188*
NP-40 (0.01%)	2909
Triton X-100 (0.01%)	4310
Tween 40 (0.01%)	2804
Tween 80 (0.01%)	2817

* Average of duplicate enzyme assays. Standard assay conditions were used with 10 μCi of dTTP (11.4 Ci/mmol), approximately 14 μg of viral protein, and 1 hr of incubation.

TABLE 2. *DNA polymerase activity of murine sarcoma virus (Harvey) and murine sarcoma virus (Moloney).*

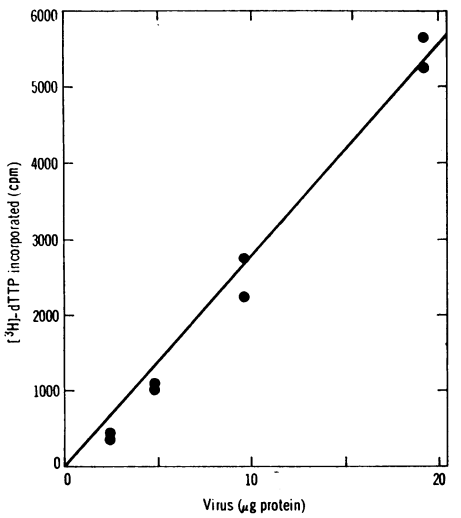
Virus	Time (min)	Incorporation of [3H]dTTP (cpm*)
MSV(H)	0	49
	60	2819
MSV(M)	0	223
	60	15026

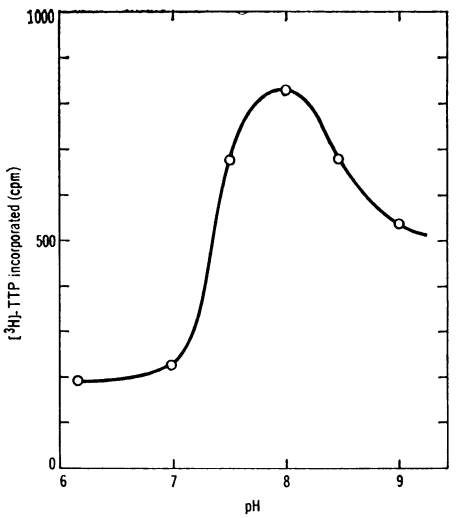
* Average of duplicate enzyme assays. Standard assay conditions were used with 10 μCi of [3H]dTTP (11.4 Ci/mmol) and 9.2 μg of MSV(H) or 9.6 μg of MSV(M).

(1) *Relationship between DNA synthesis and virus concentration:* The amount of DNA synthesized by NP-40-activated virus is proportional to the concentration of viral protein from 2.4 to 19.2 μg of protein per assay (Fig. 2).

(2) *pH and temperature:* The pH optimum for the DNA polymerase from MSV(H) is about 8.1 (Fig. 3). Maximal enzyme activity for NP-40-activated MSV(H) occurs at 37°C (Fig. 4). Incorporation is decreased at 40, 45, and 50°C (Fig. 4).

(3) *Deoxyribonucleoside triphosphates:* All four deoxyribonucleoside triphosphates are required for polymerase activity. Omission of one of the four deoxyribonucleoside triphosphates reduced incorporation of dTTP nearly completely (Table 3). The incorporation of α-^{32}P-labeled dTTP, dATP, dGTP, and dCTP is linear for at least 90 min (Fig. 5) (the different incorporation rates reflect different concentrations of labeled nucleotides in the assay mixture and,





(*Left*) FIG. 2.—Linear relationship between virus concentrations and MSV DNA polymerase activity. The standard assay was used with 0.01% NP-40 and 10 μCi of [^{3}H]dTTP (11.4 Ci/mmol).

(*Right*) FIG. 3.—Effect of pH on the incorporation of dTTP by the MSV DNA polymerase. The standard assay was used with 0.01% NP-40, 10 μCi of [^{3}H]dTTP (4.3 Ci/mmol) and 2.96 μg of viral protein. The following buffers, at a concentration of 40 mM, were used: Tris·HCl buffer at pH 7.0, 7.5, 8.10, 8.45; phosphate buffer at pH 6.15, and Tris acetate buffer at pH 9.0.

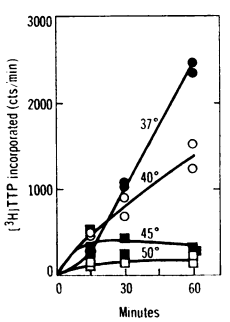


FIG. 4.—Effect of temperature on NP-40-activated incorporation of dTTP by the DNA polymerase from MSV. The standard assay was used with 10 μCi of [^{3}H]dTTP (11.4 Ci/mmol) and 7.2 μg of viral protein.

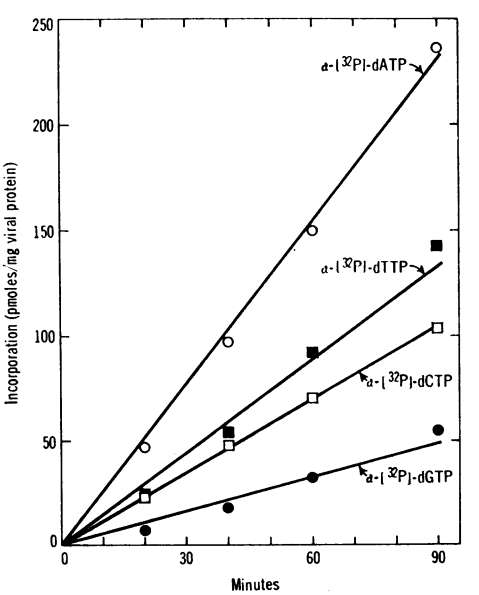


FIG. 5.—Incorporation of α-^{32}P-labeled deoxyribonucleoside triphosphates into DNA by the MVA DNA polymerase. The standard assay was used with 0.01% NP-40, 0.1 mM of three unlabeled deoxyribonucleoside triphosphates, and one labeled with ^{32}P, and 6.3 μg of viral protein in 300 μl final volume. Aliquots (50 μl) were assayed at 0, 20, 40, 60, and 90 min. The following concentrations of labeled precursors was used: (1) [α-^{32}P]dATP, 2.5 μCi/50 μl (0.88 mCi/mmol); (2) [α-^{32}P]dGTP, 2.5 μCi/50 μl (4 Ci/mmol); (3) [α-^{32}P]dTTP, 1.78 μCi/50 μl (3.8 Ci/mmol); (4) [α-^{32}P]dCTP, 3.9 μCi/50 μl (4.0 Ci/mmol).

perhaps, the base composition of the template). Polymerase activity is not affected by 10 mM phosphate but is fully blocked by 10 mM pyrophosphate.

(4) *Metal ions and reducing agent:* Divalent cations are essential for polymerase activity. Omission of Mg^{2+}, or the addition of 10 mM EDTA, completely inhibits incorporation of dTTP (Table 4). The optimal concentration of Mg^{2+} is 1–2.5 mM. Mn^{2+} effectively replaces Mg^{2+}, increasing incorporation 50% (Table 4, Expt. 2). This is surprising since Mn^{2+} is generally a poor substi-

TABLE 3. *Deoxyribonucleoside triphosphate requirement for DNA synthesis.*

Components	Incorporation of [^{3}H]dTTP (cpm)
Complete system*	3561†
Minus dATP	248
Minus dGTP	89
Minus dCTP	386

* Standard assay conditions were used with 0.01% NP-40, 10 μCi of [^{3}H]dTTP (11.4 Ci/mmol), and 12.6 μg of viral protein.

† Average of duplicate enzyme assays. Zero time incorporation, 140 cpm was subtracted.

TABLE 4. *Requirements for divalent cations for DNA synthesis.*

Components	Incorporation of [^{3}H]dTTP (pmol/mg viral protein/hr)
Expt. 1	
Complete system* (5 mM Mg^{2+}, 5 mM dithiothreitol)	229†
2.5 mM Mg^{2+}	468
10 mM Mg^{2+}	146
$-Mg^{2+}$	4
+10 mM EDTA	5
2.5 mM dithiothreitol	202
10.0 mM dithiothreitol	227
−dithiothreitol	125
Expt. 2	
Complete system (2.5 mM Mg^{2+})‡	193
1.0 mM Mg^{2+}	250
0.5 mM Mg^{2+}	116
$-Mg^{2+}$ + 5.0 mM Mn^{2+}	296
$-Mg^{2+}$ + 2.5 mM Mn^{2+}	311
$-Mg^{2+}$ + 1.0 mM Mn^{2+}	382
$-Mg^{2+}$ + 0.5 mM Mn^{2+}	351

* Standard assay conditions were used with 0.01% NP-40, 5 mM $MgCl_2$, 0.023 mM [^{3}H]-dTTP (4.3 Ci/mmol), and 2.96 μg of viral protein.

† Average of duplicate enzyme assay. Counts after 1 hr were 1018 cpm above a zero time incorporation of 148 cpm.

‡ Standard assay conditions were used with 0.01% NP-40, 2.5 mM $MgCl_2$, 0.025 mM [^{3}H]dTTP (4.8 Ci/mmol) (this lot of [^{3}H]-dTTP is different from that in Expt. 1), and 2.96 μg viral protein.

TABLE 5. *Effect of RNase on the template.*

	Incorporation of [^{3}H]dTTP (cpm*)
Complete system†	3652
+RNase (100 μg/ml)	991

* Average of duplicate enzyme assays with 10 μCi of [^{3}H]dTTP (11.4 Ci/mmol) and 9.6 μg of viral protein.

† The virus was incubated with pancreatic RNase for 45 min in the presence of NP-40 before the standard DNA polymerase assay.

tute for the Mg^{2+} requirement of DNA polymerases of animal cells.[9] Omission of dithiothreitol decreases incorporation 50%; no differences in incorporation were observed when the concentration of dithiothreitol was 2.5, 5, or 10 mM.

Nature of the template: Two lines of evidence suggest that viral RNA is the template for the DNA polymerase of MSV: (1) *RNase destroys polymerase activity:* Incubation of NP-40-treated MSV(H) with RNase reduced incorporation by 73% (Table 5).

(2) *RNA tumor viruses contain no DNA?* RNA tumor viruses contain 70S viral RNA which is converted to 35S RNA by various denaturing agents;[1, 10] the

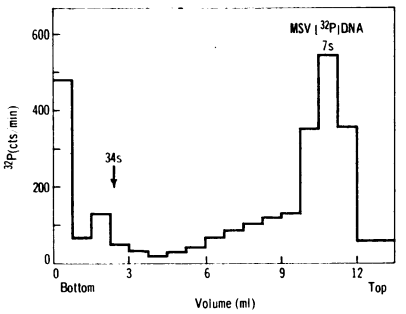
molecular weight of viral RNA estimated from these S values ranges from 3 to 13 × 10^6. Experiments which could exclude the presence of a small amount of DNA, e.g., 250,000–500,000 daltons, have not been reported. We attempted to determine whether MSV may contain a DNA template for the DNA polymerase reaction. Four millicuries of [^{3}H]thymidine (17.8 Ci/mmol) was added to 2 liters of MEH cells in suspension (2.0 × 10^5 cells/ml). After 24 hr at 37°C, when the cell number had doubled, the culture was centrifuged, virus was isolated from the medium, and cell DNA was extracted from the pellet.[11] From the specific activity of the cell DNA (10,960 cpm/μg), and if we assume a doubling of the DNA content, we estimate that newly synthesized DNA would have a specific activity of about 22,000 cpm/μg. Virus (1.5 ml) isolated from the medium contained 926 μg of viral proteins/ml and 6984 cpm/ml, thus a specific activity of 7.5 cpm/μg of viral protein. The specific activity was reduced to 3 cpm/μg by treatment with DNase, sucrose density gradient centrifugation, and two cycles of solubilization in alkali and precipitation with perchloric acid. From the values of 3–7 cpm/μg, and if we assume a particle weight of 450 × 10^6 daltons, we estimate that not more than 50,000–100,000 daltons of DNA can be present in a MSV particle.

Properties of the product: The following experiments show that the product has properties of DNA with regard to DNase digestion, stability in alkali, sedimentation in alkaline sucrose gradients, and buoyant density in isopycnic CsCl gradients. (1) *The product is hydrolyzed by DNase but not by RNase:* The [^{3}H]dTTP labeled polymerase product was rendered acid-soluble by DNase or snake venom phosphodiesterase (70–80%) but not by RNase (Table 6). (2) *The product is stable in alkali:* There is no reduction in the acid-precipitable ^{3}H-product after treatment with 0.2 N alkali for 20 min at 80°C, conditions which convert RNA, but not DNA, to an acid-soluble form. (3) *The product sediments in alkaline sucrose gradients:* The labeled product was centrifuged in a 5–20% alkaline sucrose gradient together with a 34S adenovirus type 7 DNA marker.[12] The product synthesized by NP-40-activated MSV(H) sediments at 7 S (Fig. 6), corresponding to single-stranded DNA of molecular weight 200,000–250,000.[13] (4) *The product has a buoyant density like that of DNA:* Preparations of labeled product were denatured and centrifuged in CsCl density gradients (Fig. 7). The [^{3}H]MSV product has a much broader peak than marker adenovirus type 7 [^{32}P]DNA, as expected from the differences in molecular weight. Based on the known buoyant density of

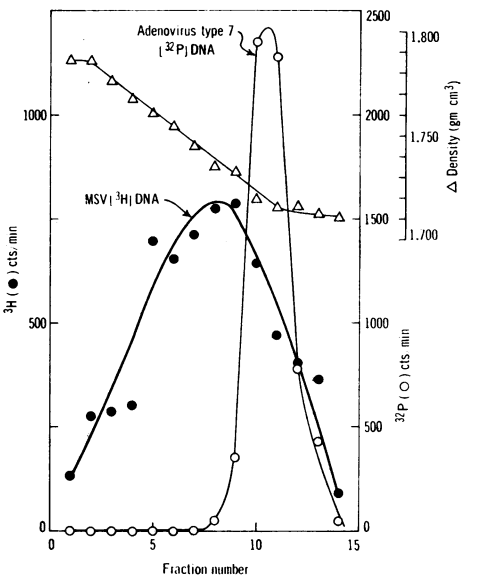
TABLE 6. *Effect of nucleases on the product of the MSV(H) polymerase reaction.*

Treatment	Incorporation of [^{3}H]dTTP (cpm)*
None	3287
RNase, 100 μg/ml	3358
RNase, 1 mg/ml	3224
DNase, 100 μg/ml	1100
DNase, 1 mg/ml	993
Snake venom phosphodiesterase, 100 μg/ml	1037
Snake venom phosphodiesterase, 400 μg/ml	658
RNase, 100 μg/ml + DNase, 100 μg/ml	1263
RNase, 1 mg/ml + DNase, 1 mg/ml	960

* Average of duplicate enzyme assays with 10 μCi of [^{3}H]dTTP (11.4 mCi/mmol) and 9.6 μg of viral protein. After 1 hr of incubation at 37°C, nucleases were added and incubation was continued for 1 hr at 37°C.



(*Above*) FIG. 6.—Zonal centrifugation of the MSV polymerase product in alkaline sucrose gradients. 100 μl of the product of polymerase assay with 5 μCi of [α-^{32}P]dATP (0.88 mCi/mmol) and 0.01% NP-40 was layered over a 5–20% alkaline sucrose gradient and centrifuged at 4°C in the Spinco SW41 rotor for 10 hr at 38,000 rpm. Fractions were collected and the alkali stable, acid-insoluble, ^{32}P-labeled product was counted. Adenovirus type 7, labeled with [^{14}C]thymidine, was centrifuged as a 34S DNA marker.



(*Right*) FIG. 7.—CsCl density gradient equilibrium centrifugation of the product of the MSV polymerase reaction. 50 μl of a 60-min reaction product of the standard enzyme assay with 0.01% NP-40, 10 μCi of [^{3}H]dTTP (11.4 Ci/mmol), and 13 μg of viral protein of MSV(H) were treated with 20 μl of 2 N NaOH at 80°C for 20 min and neutralized with 20 μl of 3 M NaH_2PO_4. The sample was centrifuged in a CsCl density gradient at 42,000 rpm for 30 hr with marker adenovirus type 7 [^{32}P]DNA. Fractions were collected and the acid-precipitable radioactivity was measured.

adenovirus type 7 DNA (ρ = 1.713), we estimate that the mean buoyant density of the product shown in Fig. 7 and in other determinations of 30- and 90-min products is 1.736–1.738, a value consistent with the product being DNA.

Discussion. The presence of a unique enzyme, an RNA-dependent DNA polymerase, in RNA tumor viruses raises many provocative questions concerning the mechanism of enzyme action, the nature of the template and the product, the function of the DNA product in virus replication and cell transformation, and the possible role of information flow from RNA to DNA in normal cell function.

Evidence that viral RNA is the template is provided by the decrease in polymerase activity of disrupted virions upon treatment with RNase and the absence of an appreciable amount of DNA in the MSV virion. But decisive proof that viral RNA is the template requires the demonstration of (1) RNA template dependency of purified MSV polymerase and (2) hybridization of the DNA product with viral RNA (see *Note added in proof*).

The product of the reaction is alkali-stable DNA, digestible by DNase, sedimentating at 7 S in alkaline sucrose gradients, and possessing a mean buoyant density of 1.736 in CsCl. Although isolated MSV DNA is much smaller than viral RNA, the possibility exists that the entire viral genome is copied, and

that the smaller DNA molecules are produced by contaminating nucleases, or possibly represent intermediates in DNA synthesis.[14]

What is the biological function of the viral DNA product? We speculate on several possibilities. (1) As a DNA·RNA hybrid, viral DNA protects the genetic information of viral RNA from nucleases. (2) Viral DNA may bind to specific regions of cellular DNA and modify the activity of cellular genes involved in virus replication. It might be significant that 4% of the RNA sequences in RNA tumor viruses hybridizes with the DNA of normal uninfected cells.[1] (3) Viral DNA may be copied from the entire viral RNA genome and be integrated into cellular chromosomes where it serves as a template for the synthesis of progeny viral RNA, and as a continuous source of information for the maintenance of the neoplastic phenotype of the cell.

Possibly information flow from RNA to DNA plays an important role in normal cell development. Huebner[15] proposes that cellular genes carrying genetic information of RNA tumor viruses are expressed in carcinogenesis, and recent studies indicate that some RNA tumor virus information is also expressed during embryonic development in the mouse.[16] Further studies on virus replication and cell transformation by RNA tumor viruses offer unique opportunities to understand molecular mechanisms involved in cellular growth and neoplasia, and may provide important leads towards an understanding of normal gene expression during embryonic development and cell differentiation.

Note added in Proof. We have recently described two lines of evidence which show that viral RNA is the template for the MSV DNA polymerase: (1) viral RNA·DNA hybrid molecules are formed by the MSV DNA polymerase and (2) the DNA product formed by the MSV DNA polymerase hybridizes with viral RNA (Rokutanda, Rokutanda, Green, Fujinaga, Ray, and Gurgo, *Nature*, in press).

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Abbreviations: MSV, murine sarcoma virus; NP-40, Nonidet P-40.

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‡ On leave from the Aichi Cancer Center Research Institute, Nagoya, Japan.

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