Poly(2′-*O*-methylcytidylate)·Oligodeoxyguanylate as a Template for the Ribonucleic Acid Directed Deoxyribonucleic Acid Polymerase in Ribonucleic Acid Tumor Virus Particles and a Specific Probe for the Ribonucleic Acid Directed Enzyme in Transformed Murine Cells†

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ABSTRACT: The potential of the 2′-*O*-methylated polynucleotides, poly(2′-*O*-methyladenylate), poly(2′-*O*-methylinosinate), poly(2′-*O*-methyluridylate), and poly(2′-*O*-methylcytidylate) [poly(Cm)], as specific templates for viral RNA directed DNA polymerase was evaluated. Of the four homopolymers, poly(Cm) complexed to a complementary oligodeoxyribonucleotide was the most effective template for the purified RNA directed DNA polymerase from virions of avian myeloblastosis virus. The optimal metal ion concentrations for poly(Cm)·oligo(dG)-directed DNA synthesis catalyzed by avian myeloblastosis virus DNA polymerase were 0.15 mM Mn^{2+} and 5–10 mM Mg^{2+}. The rate of synthesis was 40-fold greater with Mn^{2+} than with Mg^{2+}. Poly(Cm)·oligo(dG) was an effective template for the RNA directed DNA polymerase of RNA tumor viruses of avian, murine, feline, and primate origin. None of the 2′-O-methylated homopolymers served as templates for *Micrococcus luteus* DNA polymerase or *Escherichia coli* DNA polymerase I. At least four different DNA polymerases were purified by DEAE-cellulose and phosphocellulose chromatography, and Sephadex G-200 gel filtration from extracts of a clonal line of mouse 3T6 fibroblast cells transformed by the Harvey strain of murine sarcoma-leukemia virus. Among the four enzymes, only the DNA polymerase that was identified as a viral RNA directed DNA polymerase was able to use poly(Cm)·oligo(dG) as template. Nontransformed mouse 3T6 cells contained no detectable DNA polymerase activity which responded to poly(Cm)·oligo(dG) as template.

Until recently, evidence for the intracellular function of the RNA directed DNA polymerase of RNA tumor viruses has been mainly indirect. Evidence is beginning to accumulate, however, which indicates that in RNA tumor virus replication and cell transformation, viral RNA directed DNA polymerase functions in the synthesis of virus-specific DNA. For example, (i) reassociation kinetic analyses (Varmus *et al.*, 1973) and *in situ* cytological hybridization experiments (M. Loni and M. Green, submitted for publication) using the DNA product of RNA tumor virus RNA directed DNA polymerase have demonstrated the presence of viral specific gene sequences in the DNA of virus-transformed, but not normal, animal cells, and (ii) temperature-sensitive mutants of Rous sarcoma virus have been isolated (Linial and Mason, 1973) in which the temperature sensitive lesion appears to be located in the RNA directed DNA polymerase (I. Verma, W. Mason, and D. Baltimore, personal communication). The role of the RNA tumor virus and its DNA polymerase in the etiology of human cancer remains to be elucidated. Spiegelman and his colleagues have searched in human tumors for RNA molecules related to those found in RNA tumor viruses by sequence, size, and by association with an RNA directed DNA polymerase (for review, see Baxt and Spiegelman (1972)). The techniques used involve RNA–DNA hybridization, and rate–zonal centrifugation to simultaneously detect a 70S RNA template associated with an RNA directed DNA polymerase in extracts of human tumors (Gulati *et al.*, 1972). The "simultaneous detection" technique has several disadvantages among which are the necessity of using large amounts of highly labeled radioactive DNA precursors to synthesize small quantities of 70S RNA–labeled DNA complex, the possible loss of unknown quantities of putative 70S RNA–labeled DNA complex due to nucleolytic cleavage of the 70S RNA during incubation of crude cellular extracts, and the necessity of deproteinizing and processing the 70S RNA–DNA product on sucrose gradients. The technique has the advantage of simultaneously detecting both enzyme and template and yielding a labeled DNA product whose sequences can be analyzed, and is the only technique presently available for analyzing large numbers of human tissues for RNA directed DNA polymerase. A synthetic probe which is both resistant to nuclease and is a specific template for viral RNA directed DNA polymerase would not be subject to these difficulties. Moreover, the use of synthetic homopolymer templates which generally amplify DNA polymerase activity many fold (Spiegelman *et al.*, 1970b) would provide a much higher sensitivity of detection.

Recently, we found that certain single-stranded polyribonucleotides methylated at the 2′ position exhibited some intrinsic template specificity for bacterial DNA dependent RNA polymerase (Gerard *et al.*, 1972). Pyrimidine-containing 2′-O-methylated polynucleotides served as templates for the enzyme, but purine-containing 2′-O-methylated homopolymers did not. Both purine- and pyrimidine-containing DNA and RNA homopolymers were efficient tem-

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plates for the bacterial RNA polymerase. We therefore decided to test poly(2′-*O*-methylribonucleotides) complexed to complementary oligodeoxyribonucleotides as template primers for viral, bacterial, and cellular DNA polymerases, with the hope that a template specific for viral RNA directed DNA polymerase might be found. As an initial test system, we fractionated the DNA polymerase species present in a clonal line of mouse 3T6 cells and in the same cell line after transformation by the Harvey strain of murine sarcoma-leukemia virus. We report here that one of these polymers, poly(Cm), is specific for the viral RNA directed DNA polymerase in transformed murine cells.

Materials and Methods

Reagents. RNase A (Type IX) from Sigma Chemical Co. was heated at 80° for 10 min before use. Calf thymus DNA (Type IV) and *Escherichia coli* DNA (Type VIII) were from Sigma Chemical Co. Calf thymus DNA was activated by the procedure of Aposhian and Kornberg (1962), and DNA was denatured at a concentration of 250 μg/ml by heating at 100° for 10 min followed by quick cooling. Spectral grade formamide from Eastman was extracted with ether, aerated with N_2, and stored at −70°. The following is a list of materials and their source: [^{3}H]TTP, [^{3}H]dCTP, [^{3}H]dATP, [^{3}H]dGTP, and [α-^{32}P]dGTP, New England Nuclear; Whatman DEAE-cellulose (DE-52), phosphocellulose (P-11), and DEAE-cellulose (DE-81) paper discs, Reeve Angel; deoxyribonucleoside 5′-triphosphates (TTP, dGTP, dATP, dCTP) and dithiothreitol, Sigma Chemical Co.; Sephadex G-200, superfine, Pharmacia; synthetic polyribonucleotides and polydeoxyribonucleotides, and partially purified *Micrococcus luteus* DNA polymerase, P-L Biochemicals; nitrocellulose membrane filters (Type B-6), Schleicher & Schuell; oligo(dT), oligo(dC), oligo(dG), and oligo(dA), each 12–18 nucleotides, Collaborative Research, Inc. Poly(2′-*O*-methylribonucleotides) were prepared according to published procedures (Rottman and Heinlein, 1968; Rottman and Johnson, 1969; Dunlap *et al.*, 1971), with the exception of poly(2′-*O*-methylinosinate) [poly(Im)] (Rottman *et al.*, 1974).

Analytical Methods. Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The concentration of 70S RNA and native calf thymus and *E. coli* DNA was determined spectrophotometrically in 0.01 M Tris-HCl (pH 8.0)–0.1 M NaCl based on the extinction coefficient $E^{1\%}_{260}$ 200. The molar extinctions used to determine the homopolymer concentrations were those of Ts'o *et al.* (1962) and Bollum (1966). Concentrations of templates are given as concentration of mononucleotides. The mean value of the sedimentation coefficients determined by centrifugation through sucrose density gradients (pH 8.0) for poly(2′-*O*-methyladenylate) [poly(Am)], poly(2′-*O*-methyluridylate) [poly(Um)], poly(2′-*O*-methylcytidylate) [poly(Cm)], poly(Im), and poly(dT) were 9 S, 11 S, 4 S, 4 S, and 4 S, respectively.

Viruses. The Moloney and Harvey strains of murine sarcoma-leukemia virus, M-MSV(MLV) and H-MSV(MLV),[1] were grown and purified as previously described (Green *et al.*, 1970). RD-feline leukemia virus and RD-114 were grown in the RD-feline leukemia virus and RD-114 cell lines provided by R. McAllister, and purified as described by Green *et al.* (1970). Feline sarcoma-leukemia virus (Gardner), murine leukemia virus (Rauscher) (R-MLV), and Rous sarcoma virus (Schmidt-Ruppin) were purchased from Electro-Nucleonics Lab., Inc. Avian myeloblastosis virus, BAI strain A (AMV), was generously supplied by Dr. J. W. Beard, and was purified as described by Grandgenett *et al.* (1973).

Preparation of AMV DNA Polymerase. RNA directed DNA polymerase from AMV was purified through the phosphocellulose stage as previously described (Grandgenett *et al.*, 1973). The two subunit enzyme (αβ) was used in this study.

Preparation of 70S RNA. Viral 70S RNA was isolated from purified AMV, M-MSV(MLV), and RD-114 virus by phenol extraction and zonal centrifugation in sucrose gradients (Tsuchida *et al.*, 1972).

DNA Polymerase Assays. All incubations were at 37°. The details of each assay are described in the figure legends and table footnotes. When ^{3}H-labeled DNA precursors were used, the reaction was stopped by the addition of EDTA to a final concentration of 20 mM. The amount of polymer product formed was determined by assay on Whatman DEAE-cellulose paper discs (Grandgenett *et al.*, 1972). When ^{32}P-labeled precursors were used, the reaction was terminated by the addition of 150 μl of cold 1 N perchloric acid/100 μl of reaction mixture. Calf thymus DNA, 70 μl of 1 mg/ml, and ATP, 25 μl of 25 mg/ml, were then added. After 5 min at 4°, the mixture was centrifuged at 8000 rpm for 5 min. After removal of the supernatant, the precipitate was dissolved in 100 μl of 0.2 N NaOH. The precipitation with perchloric acid and dissolution with NaOH were repeated once; the DNA product was finally precipitated with 2 ml of cold 10% Cl_3-CCOOH, collected on nitrocellulose membrane filters, and washed with 15 ml of 5% Cl_3CCOOH–1% sodium pyrophosphate. The radioactivity on both types of dried filters was monitored in scintillation fluid containing 4 g of 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene/l. of toluene.

Preparation of Nucleases. Nuclease specific for single-stranded DNA or RNA was purified from conidia (Miles) of *Neurospora crassa* by the procedure of Rabin *et al.* (1971), omitting the preparative electrophoresis.

S_1 single-strand nuclease was prepared from Diastase powder of *Asperigillus oryzae* (Sigma) by the procedure described by Sutton (1971).

Preparation of [^{3}H]DNA for Hybridization to Viral 70S RNA. DNA was synthesized from both AMV and M-MSV(MLV) 70S RNA in a reaction mixture (1.0 ml) containing 20 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 30 mM NaCl, 0.2 mM $MnCl_2$, 100 μM each of dATP, dCTP, and dGTP, 20 μM [^{3}H]TTP (27,000 cpm/pmol), 100 μg/ml of actinomycin D, either 19 μg/ml of AMV 70S RNA or 9 μg/ml of M-MSV(MLV) 70S RNA, and 300 μl of Sephadex G-200 purified peak II (see Table IV). Incubation was terminated after 90 min by the addition of 100 μl of 0.2 M EDTA. Sodium dodecyl sulfate and 0.15 M NaCl–0.015 M sodium citrate (SSC) were added to final concentrations of 0.5% and 1 ×, respectively, and the mixture was extracted twice with equal volumes of 1 × SSC saturated phenol and chloroform–isoamyl alcohol (24:1, v/v). After two extractions with 2 volumes of ether, the product was dialyzed overnight against 2 l. of 0.1 × SSC. The [^{3}H]DNA (20 cpm of [^{3}H]TMP/pg) was then treated with 0.2 N NaOH for 20 min at 80° followed by 6 hr at 37° to hydrolyze associated RNA. The pH was then brought to 7.0 by the addition of NaH_2PO_4.

Nucleic Acid Hybridization for Determination with Nu-

[1] The abbreviations used that are not listed in *Biochemistry 9*, 4022 (1970) are: M-MSV(MLV) and H-MSV(MLV), the Moloney and Harvey strains of murine sarcoma-leukemia virus; R-MLV, Rauscher murine leukemia virus; AMV, avian myeloblastosis virus.

cleases. AMV [^{3}H]DNA (700 pg) prepared as already described was annealed with viral 70S RNA (1.4 μg) in a reaction mixture (150 μl) containing 0.72 M NaCl, 0.2 mM EDTA, 0.01 M sodium piperazine-*N*,*N*′-bis(2-ethane sulfonate) (pH 6.7), and 0.07% sodium dodecyl sulfate at 68° for 24 hr. The hybridization mixture was then split into two portions: 50 μl was used directly to assay for resistance to S_1 nuclease, and 100 μl was mixed with 5 μl of denatured *E. coli* DNA (250 μg/ml), dialyzed for 24 hr against three 1-l. changes of 0.01 M Tris-HCl (pH 8.0), and 70 μl was used to assay for resistance to *Neurospora* nuclease.

Tissue Culture Cells. Uninfected mouse 3T6 fibroblast cells (clone 91) and the transformed and chronically producing cell line derived from 3T6 (clone 91) by infection with the Harvey strain of the murine sarcoma-leukemia complex (H-MSV(MLV)), 1798 (Salzberg *et al.*, 1973), were grown in 250-ml Pfizer bottles at 37° in Eagle's minimal essential medium with 10% calf serum.

Purification of the DNA Polymerases from 3T6 and 1798 Cells and from H-MSV(MLV). Unless otherwise stated, all steps were carried out at 4°. Buffer A is 50 mM Tris-HCl (pH 7.8), 500 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 20% glycerol. Buffer B is 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol.

Preparation of Cellular and Viral Extracts. For the preparation of extracts from 3T6 or 1798 cells, the cells in 15–40 Pfizer bottles were washed three times with phosphate-buffered saline, harvested by scraping, pelleted by centrifugation at 3000 rpm for 15 min, resuspended in 5 volumes of 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 0.1 mM EDTA, and recentrifuged. The cell pellet obtained (10–30 g of packed cells) was stored at −70°. Frozen cells were thawed, suspended in 2.5 volumes of buffer A per gram of packed cells, and sonicated until completely disrupted (three to five 10-sec bursts with a Blackstone Ultrasonic Probe set at 30% of full power). An equal volume of saturated ammonium sulfate in 50 mM Tris-HCl (pH 7.8) and 1 mM EDTA was then slowly added with stirring to the sonicate. After stirring for 30 min, the suspension was centrifuged at 10,000 rpm for 30 min, and the resulting pellet was resuspended in 10–30 ml of buffer A. This suspension was made 0.5% in Triton X-100, incubated at 37° for 15 min, and then centrifuged at 100,000*g* for 60 min. The supernatant was decanted and the pellet was reextracted with 5–15 ml of buffer A. After centrifuging the reextracted pellet at 100,000*g* for 60 min, the supernatants (S-100 fraction) were combined and used for isolation of DNA polymerases. The S-100 fraction could be stored at −70° for at least 1 month without loss of DNA polymerase activity.

H-MSV(MLV) (7 mg of protein at 600 μg/ml) was dialyzed against 1 l. of buffer B for 2 hr before use. Triton X-100 was then added to the virus to a final concentration of 0.5%, and the suspension was incubated at 37° for 15 min. The lysed virus was immediately applied to a DEAE-cellulose column.

Chromatography on DEAE-Cellulose. The 3T6 or 1798 S-100 fraction was dialyzed for 8 hr against 2 l. of buffer B. After centrifugation at 10,000 rpm for 10 min, the small amount of pelleted material was discarded and the dialyzed S-100 fraction (protein concentration, 7 mg/ml) was applied to a 2.5 × 20 cm DEAE-cellulose column equilibrated in buffer B. The column was eluted with buffer B containing 0.3 M KCl, and the peak of material absorbing at 280 nm was collected, pooled (DEAE pool fraction), and dialyzed against 2 l. of buffer B for 3 hr. The protein concentration in the dialyzed DEAE pool fraction was 600 μg/ml, and the DNA polymerase activity recovered, assuming the activity in the S-100 fraction to be 100%, ranged from 50 to 60% with activated calf thymus DNA and from 80 to 100% with poly(A)·oligo(dT) as templates.

Lysed H-MSV(MLV) virus was applied to a 1.5 × 6 cm DEAE-cellulose column. The material absorbing at 280 nm was eluted, pooled, and dialyzed as described above.

Chromatography on Phosphocellulose. The dialyzed DEAE pool fraction from either 3T6 or 1798 cells was applied at a flow rate of 0.5 ml/min to a 1.5 × 23 cm phosphocellulose column equilibrated in buffer B. All detectable DNA polymerase activity adsorbed to the column. Following a wash with 60 ml of buffer B with 0.1 M KCl, the column was eluted with a 200-ml continuous gradient of 0.1–0.8 M KCl in buffer B. Fractions of 3.6 ml were collected and aliquots were assayed for DNA polymerase activity with activated calf thymus DNA, poly(A)·oligo(dT), and poly(Cm)·oligo(dG) as templates. The salt concentration in each fraction was determined conductometrically. The total recovery of DNA polymerase activity ranged from 70 to 90%.

The dialyzed H-MSV(MLV) DEAE pool fraction (protein concentration, 130 μg/ml) was applied at a flow rate of 0.5 ml/min to a 0.9 × 16 cm phosphocellulose column equilibrated in buffer B. Following a wash with 10 ml of buffer B with 0.1 M KCl, the column was eluted with a 100-ml continuous gradient of 0.1–0.8 M KCl in buffer B. Fractions of 2.2 ml were collected and aliquots were assayed with the three templates described above. Recovery of enzyme activity was 100%.

Concentration of Phosphocellulose Eluate. The fractions containing the bulk of the enzyme activity from the phosphocellulose columns of 1798 and H-MSV(MLV) were pooled (see Figure 1 for details), diluted two- to threefold in buffer B, and applied to phosphocellulose columns 0.9 × 1–6 cm in size. Enzyme activity was eluted in a small volume (2 to 5 ml) with 0.6 M KCl in buffer B. Recovery of enzyme activity was 60–70%.

Chromatography on Sephadex G-200. The concentrated phosphocellulose eluates from 1798 and H-MSV(MLV) were separately loaded on a 1.5 × 85 cm column of Sephadex G-200, superfine, equilibrated with 200 mM KCl in buffer B. Samples were loaded in a volume of 1 ml in buffer A. The column was developed at a flow rate of 1.7 ml/hr with 200 mM KCl in buffer B; 1-ml fractions were collected and assayed for DNA polymerase activity with activated calf thymus DNA, poly(A)·oligo(dT), and poly(Cm)·oligo(dG).

Results

Poly(2′-O-methylribonucleotides) as Templates for Purified AMV DNA Polymerase and Bacterial DNA Polymerase. To establish that 2′-O-methylated homopolymers can serve as templates for viral RNA directed DNA polymerase, we tested poly(Am), poly(Um), poly(Cm), and poly(Im) for activity with the αβ form of DNA polymerase from AMV (Grandgenett *et al.*, 1973). With the purified enzyme, none of the 2′-O-methylated homopolymers alone stimulated polymerization of the complementary [^{3}H]deoxyribonucleoside triphosphate (data not shown). The results in Table I show, however, that addition of a complementary oligodeoxyribonucleotide (12–18 nucleotides in length) to each of the poly(2′-*O*-methylribonucleotides) stimulated incorporation with poly(Cm), but not appreciably with poly(Am), poly(Um), or poly(Im). Oligo(dG) alone did not support poly(dG) synthesis by AMV DNA polymerase (data not shown); the rate of the reaction with poly(Cm)·oligo(dG) was greater with Mn^{2+} than with Mg^{2+} (Table I). Results similar to these were

TABLE I: Poly(2′-*O*-methylribonucleotides) as Templates for AMV RNA Directed DNA Polymerase and *M. luteus* DNA Directed DNA Polymerase.[a]

Polynucleotides	pmoles of [^{3}H]Deoxyribonucleotide Incorporated/20 min			
	AMV DNA Polymerase		*M. Luteus* DNA Polymerase	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
Poly(Am) + oligo(dT)	0.3	2.2	<0.1	<0.1
Poly(Um) + oligo(dA)	<0.1	1.1	<0.1	<0.1
Poly(dT) + oligo(dA)			505	196
Poly(Cm) + oligo(dG)	66.5	98.1	0.2	<0.1
Poly(dC) + oligo(dG)			1160	634
Poly(Im) + oligo(dC)	<0.1	<0.1	<0.1	<0.1

[a] Reaction mixtures (0.1 ml) for assaying $\alpha\beta$ AMV DNA polymerase contained 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM dithiothreitol, either 2 mM $MgCl_2$ or 2 mM $MnCl_2$, 30 μM template and 10 μM primer, and 4 μg/ml of AMV DNA polymerase. Reaction mixtures (0.1 ml) for *M. luteus* DNA polymerase contained 50 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, either 2 mM $MgCl_2$ or 2 mM $MnCl_2$, 30 μM template and 10 μM primer, and 9 units/ml of *M. luteus* DNA polymerase. Both types of reaction mixtures also contained the appropriate ^{3}H-labeled deoxyribonucleoside triphosphate at the following concentrations: 5 μM [^{3}H]TTP (21,000 cpm/pmol), 10 μM [^{3}H]dATP (2300 cpm/pmol), 17 μM [^{3}H]-dGTP (1800 cpm/pmol), or 4 μM [^{3}H]dCTP (2300 cpm/pmol).

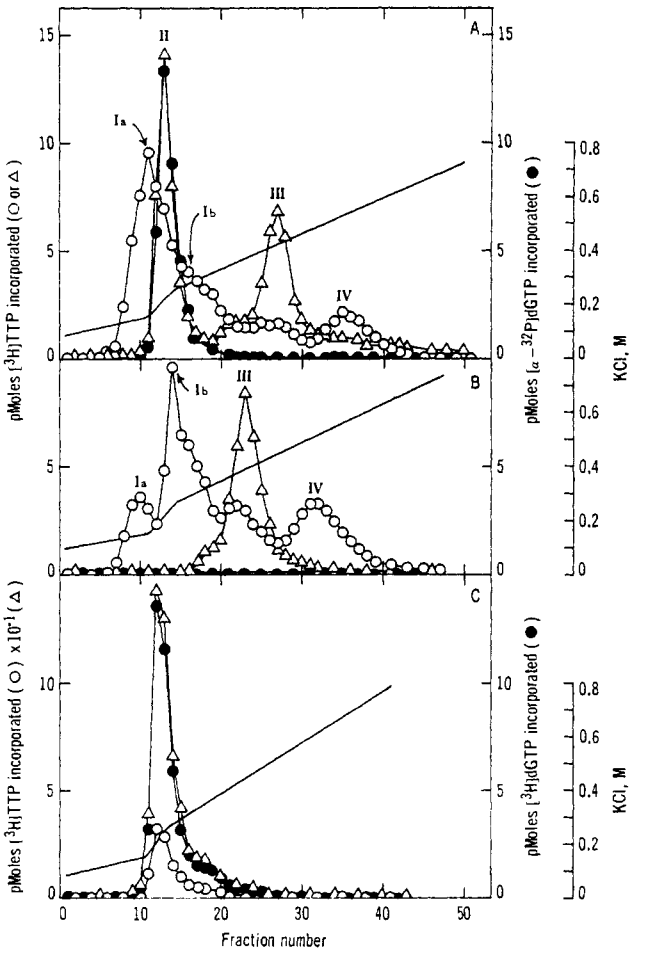

FIGURE 1: Chromatography of DNA polymerase from 1798 cells, 3T6 cells, and H-MSV(MLV) on phosphocellulose. (A) Dialyzed DEAE pool (60 mg of protein) from 1798 cells was chromatographed on phosphocellulose as described in Materials and Methods. Samples from each fraction were assayed for activity with activated calf thymus DNA (○), poly(A)·oligo(dT) (△), and poly(Cm)·oligo(dG) (●). The assay mixture (0.1 ml) with activated calf thymus DNA contained 50 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 5 mM $MgCl_2$, 2 μM [^{3}H]TTP (27,000 cpm/pmol), 100 μM each of dATP, dCTP, and dGTP, 50 μg/ml of activated calf thymus DNA, and 10 μl of sample. Incubation was for 30 min. With poly(A)·oligo(dT) as template, the assay mixture (0.1 ml) contained 50 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 0.2 mM $MnCl_2$, 2 μM [^{3}H]-TTP (27,000 cpm/pmol), 45 μM poly(A), 13 μM oligo(dT), and 10 μl of sample. The time of incubation was 30 min. Samples of 20 μl each were assayed with poly(Cm)·oligo(dG) in a reaction mixture (0.1 ml) containing 50 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 0.2 mM $MnCl_2$, 1 μM [α-^{32}P]dGTP (15,000 cpm/pmol), 52 μM poly(Cm), and 13 μM oligo(dG). Incubation was for 60 min. Fractions eluting at 0.12–0.30 M KCl (peak Ia + Ib plus peak II), at 0.33–0.47 M KCl (Peak III), and at 0.50–0.60 M KCl (peak IV) were pooled and concentrated as described in Materials and Methods. (B) Dialyzed DEAE pool (93 mg of protein) from 3T6 cells was chromatographed on phosphocellulose as described in Materials and Methods. Samples from each fraction were assayed for DNA polymerase activity as described above. (C) Dialyzed DEAE pool (3 mg of protein) from H-MSV(MLV) was chromatographed on phosphocellulose as described in Materials and Methods. Samples from each fraction were assayed for DNA polymerase activity as described above with the following exceptions: (1) with activated calf thymus DNA and poly(A)·oligo(dT) as templates, [^{3}H]TTP (3500 cpm/pmol) was 12 μM and (2) the reaction mixture with poly(Cm)·oligo(dG) was incubated for 30 min and contained 10 μM [^{3}H]dGTP (2200 cpm/pmol), 21 μM poly(Cm), 6 μM oligo(dG), and 10 μl of sample. Fractions eluting at 0.16–0.32 M KCl were pooled and concentrated as described in Materials and Methods.

obtained with a partially purified preparation (Grandgenett *et al.*, 1972) of M-MSV(MLV) DNA polymerase (data not shown): among the 2′-O-methylated homopolymers, poly(Cm)·oligo(dG) alone had template activity. Poly(Cm)·oligo(dG) was also an effective template for the single subunit form, α, of AMV RNA directed DNA polymerase (Grandgenett *et al.*, 1973). Under the assay conditions described in Table I with 0.2 mM instead of 2.0 mM $MnCl_2$ and 13 μg/ml of phosphocellulose-purified α AMV DNA polymerase, 175, 4, 1.3. and <0.1 pmol of complementary [^{3}H]-deoxyribonucleotide was incorporated in 20 min with poly(Cm)·oligo(dG), poly(Am)·oligo(dT), poly(Im)·oligo(dC), and poly(Um)·oligo(dA), respectively.

To determine if bacterial DNA polymerases can use poly(2′-*O*-methylribonucleotides) as templates, the homopolymers with appropriate primers were tested for activity with purified *M. luteus* DNA polymerase and *E. coli* DNA polymerase I (fraction VII, Jovin *et al.* (1969), a gift from I. Lehman). The results with *M. luteus* DNA polymerase are shown in Table I. None of the 2′-O-methylated polymers with appropriate primers stimulated any incorporation by *M. luteus* DNA polymerase. The enzyme did respond quite well, however, to DNA templates such as poly(dC)·oligo(dG) and poly(dT)·oligo(dA). Similarly, *E. coli* DNA polymerase I did not use the primed 2′-O-methylated homopolymers as templates (data not shown). The experiments in Table I were not carried out at the concentrations of divalent metal ions which were later found to be optimal for poly(Cm)·oligo(dG)-directed activity with $\alpha\beta$ AMV DNA polymerase (see below). In a separate experiment carried out at 0.2 mM in-

TABLE II: Poly(Cm)·oligo(dG) as a Template-primer for RNA Directed DNA Polymerase of RNA Tumor Viruses.[a]

Virus	pmoles of [^{3}H]-dGTP Incorporated/20 min	
	Mg^{2+}	Mn^{2+}
Avian myeloblastosis	9.6	155
Rous sarcoma (Schmidt-Ruppin)	9.8	169
Murine sarcoma-leukemia (Moloney)	0.7	49.8
Murine sarcoma-leukemia (Harvey)	0.4	17.8
Murine leukemia (Rauscher)	0.2	11.7
Feline sarcoma-leukemia (Gardner)	1.4	17.8
RD-Feline leukemia	0.6	9.0
RD-114	1.5	35.4

[a] Virus preparations were suspended in 10% glycerol, 50 mM Tris-HCl (pH 8.0), 200 mM KCl, 50 mM dithiothreitol, and 0.1 mM EDTA, and were lysed at 0° with 0.5% Nonidet P-40; 10 μl (3–6 μg of protein) of each lysate was assayed in a reaction mixture (0.1 ml) containing 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM dithiothreitol, 26 μM poly(Cm), 9 μM oligo(dG), 35 μM [^{3}H]dGTP (1600 cpm/pmol), and either 8 mM $MgCl_2$ or 0.15 mM $MnCl_2$.

stead of 2 mM Mn^{2+}, the pmoles of DNA product synthesized by $\alpha\beta$ AMV DNA polymerase in 20 min with poly(Cm)·oligo(dG), poly(Am)·oligo(dT), poly(Im)·oligo(dC), and poly(Um)·oligo(dA) were 450, 20, 4, and 1, respectively. At 0.2 mM Mn^{2+} with *M. luteus* DNA polymerase, the amount of product synthesized in 20 min with each of the 2′-O-methylated polymers was <0.1 pmol.

To further characterize the poly(Cm)·oligo(dG)-directed reaction, the optimal concentrations of divalent metal ion with poly(Cm)·oligo(dG) as template for $\alpha\beta$ AMV DNA polymerase were determined. Reaction mixtures (0.1 ml) contained 20 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 50 mM NaCl, 50 μM poly(Cm), 10 μM oligo(dG), 100 μM [^{3}H]dGTP (400 cpm/pmol), 4 μg/ml of $\alpha\beta$ AMV DNA polymerase, and $MgCl_2$ or $MnCl_2$ at various concentrations. Reactions were terminated after 10 min of incubation. For Mg^{2+} and Mn^{2+}, the optima were found to be 5–10 and 0.15 mM, respectively. At these concentrations, the rate of DNA synthesis with Mn^{2+} was nearly 40-fold greater than with Mg^{2+}. The optimal Mn^{2+} concentration for partially purified H-MSV(MLV) DNA polymerase with poly(Cm)·oligo(dG) was also found to be 0.15 mM.

Poly(Cm)·oligo(dG) as Template for RNA Directed DNA Polymerase of RNA Tumor Viruses. The potential of poly(Cm)·oligo(dG) as a probe for viral RNA directed DNA polymerase was further evaluated by testing its template activity with DNA polymerase from RNA tumor viruses of avian, murine, feline, and primate origin. Some of these results are shown in Table II. The DNA polymerase in detergent lysates of all the RNA tumor viruses tested effectively used poly(Cm)·oligo(dG) as a template. The rate of DNA synthesis in the presence of 0.15 mM Mn^{2+} ranged from 10- to 70-fold greater than in the presence of 8 mM Mg^{2+} for the viral DNA polymerases tested. Poly(Cm)·oligo(dG) was also found to be a good template for the DNA polymerase in Nonidet P-40 lysates of simian sarcoma virus (data not shown).

Poly(Cm)·oligo(dG) as Template for the DNA Polymerases from Transformed Mammalian Cells. We have already established that in comparing the catalytic activity of RNA tumor virus and bacterial DNA polymerases, poly(Cm)·oligo(dG) is a specific template for viral RNA directed DNA polymerase. A more stringent test of specificity can be obtained by determining the response of mammalian cell DNA polymerases to poly(Cm)·oligo(dG). For the purpose of isolating cellular DNA polymerases, we used a mouse 3T6 (clone 91) cell line as a source of control cells. The cell line derived from 3T6 (clone 91) by transformation with H-MSV(MLV), 1798 (Salzberg *et al.*, 1973), was used to determine the ability of poly(Cm)·oligo(dG) to specifically detect viral RNA directed DNA polymerase in total extracts of mammalian cells.

In order to achieve an efficient extraction and solubilization of cellular and viral enzymes, whole 3T6 and 1798 cells were disrupted and extracted with a combination of high salt and nonionic detergent (Weissbach *et al.*, 1971; Ross *et al.*, 1971; Fridlender *et al.*, 1972; Smith and Gallo, 1972). After centrifugation of the extracts at 100,000*g*, nucleic acids were removed by chromatography on DEAE-cellulose. The DNA polymerases were then fractionated by chromatography on phosphocellulose. Fractions from the phosphocellulose eluate were assayed (a) with activated calf thymus DNA, to which all known RNA tumor virus (Hurwitz and Leis, 1972) and cellular DNA polymerases will respond, (b) with poly(A)·oligo(dT) plus Mn^{2+}, to which viral RNA directed DNA polymerase (Baltimore and Smoler, 1971; Goodman and Spiegelman, 1971) and cellular R-DNA polymerase (Fridlender *et al.*, 1972; Bolden *et al.*, 1972) or DNA polymerase A (McCaffrey *et al.*, 1973) will respond, and (c) with poly(Cm)·oligo(dG).

With these three templates, extracts of the transformed 1798 cells routinely showed a total of five distinct peaks of enzyme activity on phosphocellulose (Figure 1A). Four peaks of activity eluting at 0.16 M (peak Ia), 0.28 M (peak Ib), 0.42 M (peak III), and 0.53 M (peak IV) KCl were observed with activated calf thymus DNA as template. The relative amounts of peaks Ia and Ib varied from one preparation to another. In Figure 1A, the ratio of total activity in peak Ia relative to peak Ib was 3:1. In most other preparations, the ratio of total activity in Ia relative to Ib ranged from 1:1 to 1:3. Possible reasons for this are discussed below. With poly(A)·oligo(dT) as template, a peak coincident with peak III (0.42 M KCl) and a new peak of DNA polymerase activity (peak II) eluting at 0.22 M KCl were observed (Figure 1A). Poly(A)·oligo(dT)-stimulated activity coincident with peaks Ia, Ib, and IV was not apparent. Of the DNA polymerase activities from 1798 cells eluting from phosphocellulose, only the activity at 0.22 M KCl (peak II) responded to poly(Cm)·oligo(dG) as template (Figure 1A).

Extracts of nontransformed 3T6 cells also contained DNA polymerase activities which eluted at the same salt concentration and had the same template specificities as peak Ia, peak Ib, peak III, and peak IV (Figure 1B). Missing, however, was peak II DNA polymerase. 3T6 cells contained little or no DNA polymerase activity which used poly(Cm)·oligo(dG) as template. The profile of H-MSV(MLV) DNA polymerase on phosphocellulose is shown in Figure 1C. The viral enzyme responded to all three templates and eluted as a single peak at 0.21 M KCl, similar to peak II from 1798 cells. The discontinuity in the salt gradient observed during elution of the columns described in Figure 1 was routinely obtained throughout the course of this work. The reason for this phenomena is not thoroughly understood, although it is

thought to be related to the combined use of a small volume and a steep salt gradient to elute the phosphocellulose columns. The reproducible jump in ionic strength (0.15–0.27 M KCl) served to increase the resolution obtained between the first set of peaks (peaks Ia + Ib and peak II) and peak III, and might also have caused the peak I enzyme to split into two peaks (see below).

The fractions in peak Ia + Ib plus peak II, peak III, and peak IV from 1798 cells (Figure 1A) and the peak fractions of the H-MSV(MLV) enzyme (Figure 1C) were separately pooled and concentrated on small phosphocellulose columns. The small shoulder of poly(A)·oligo(dT) activity which eluted on the low salt side (0.35 M KCl) of peak III (see Figure 1A) was pooled along with peak III. In order to further purify and characterize peak III and peak IV DNA polymerase and to resolve peak II from peak Ia + peak Ib, 1.0-ml samples from all three concentrated phosphocellulose pools were separately chromatographed in 0.2 M KCl on Sephadex G-200. As illustrated in Figure 2A, there was only one major peak of DNA polymerase activity observed with activated calf thymus DNA as template after gel filtration of the peak Ia + peak Ib plus peak II pool. From this result and because of the interdependence of the amounts of peak Ia and peak Ib observed during phosphocellulose chromatography, we will hereafter refer to this DNA polymerase as peak I. Peak I enzyme may represent a mixture of two different DNA polymerases with similar molecular dimensions or one enzyme which elutes from phosphocellulose as two peaks because of the rapid jump in ionic strength between 0.15 and 0.27 M KCl (see Figure 1A) or for some other undetermined reason. Peak I DNA polymerase did not respond to poly(Cm)·oligo(dG) as template, and was well resolved from peak II DNA polymerase by Sephadex G-200 gel filtration. Peak II DNA polymerase responded to poly(Cm)·oligo(dG), poly·oligo(dT), and to a much smaller extent to activated calf thymus DNA as templates. The elution volumes of these enzymes suggested molecular weights of about 290,000 for peak I DNA polymerase and 85,000 for peak II DNA polymerase. The DNA polymerase from H-MSV(MLV) eluted at approximately the same volume as peak II DNA polymerase from the Sephadex G-200 column (Figure 2D).

Peak III DNA polymerase from 1798 cells was found to be contaminated with large amounts of peak I enzyme and a small quantity of peak II DNA polymerase (Figure 2B), and was not resolved from peak I DNA polymerase by Sephadex G-200 chromatography. Peak III DNA polymerase was less stable than either peak I or peak II DNA polymerase during manipulations subsequent to phosphocellulose chromatography; the enzyme lost approximately 75% of its poly(A)·oligo(dT) stimulated activity during concentration and gel filtration. The molecular weight of peak III DNA polymerase was estimated to be between 200,000 and 300,000.

The elution profile of peak IV DNA polymerase on Sephadex G-200 is shown in Figure 2C. Peak IV DNA polymerase was well resolved from other contaminating DNA polymerase activities, and was estimated to have a molecular weight of 45,000.

A more quantitative study of the template specificities of the 1798 DNA polymerases was then carried out by assaying the peak fractions of each enzyme from Sephadex G-200 with six different templates. The results in Table III are expressed in terms of the relative rate of [^{3}H]deoxyribonucleotide incorporation per unit volume of enzyme, since the protein concentration of the Sephadex G-200 fractions was too low to determine accurately. The rate of reaction with the best

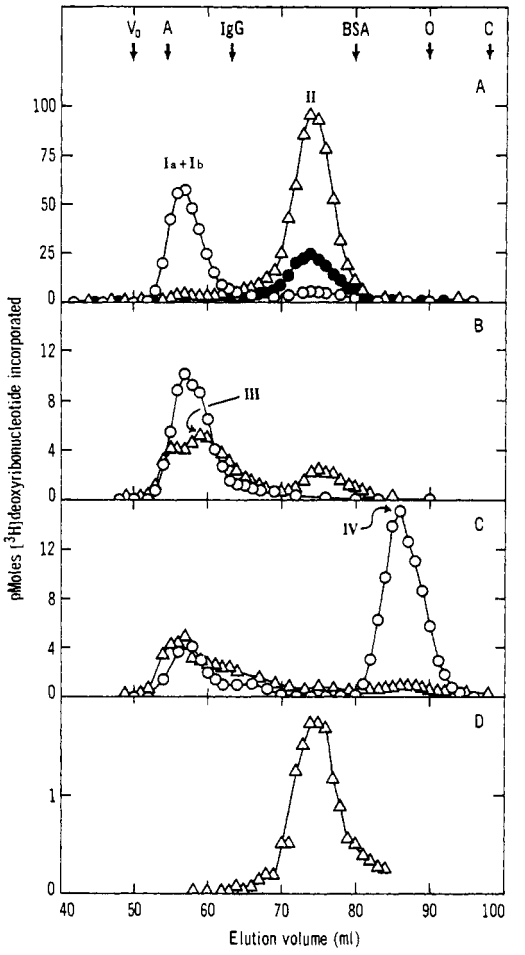

FIGURE 2: Gel filtration of the DNA polymerases from 1798 cells and H-MSV(MLV) in Sephadex G-200: (A) peak Ia + Ib and peak II (4 mg of protein), (B) peak III (1.4 mg of protein), and (C) peak IV (1.3 mg of protein) concentrated phosphocellulose eluates from 1798 cells were separately chromatographed on a Sephadex G-200 column as described in Materials and Methods. Samples (10 μl) from each fraction were assayed for activity with activated calf thymus DNA (○), poly(A)·oligo(dT) (△), and poly(Cm)·oligo(dG) (●) as described in the legend to Figure 1C. (D) H-MSV(MLV) concentrated phosphocellulose eluate (approximately 0.4 mg of protein) was chromatographed on Sephadex G-200 as described. Samples (20 μl) from fractions were assayed for activity with poly(A)·oligo(dT) as template in the same reaction mixture as described above with [^{3}H]TTP (27,000 cpm/pmol) at 5 μM. Marker proteins, detected by absorbance at 280 nm, used to calibrate the Sephadex G-200 column were γ-globulin (IgG), apoferritin (A), bovine serum albumin (BSA), ovalbumin (O), chymotrypsinogen (C), cytochrome *c*, and catalase. The void volume (V_0) was measured with blue dextran.

template for each enzyme was set at unity, and the rates determined with the other templates for each individual enzyme were expressed as a fraction of 1.0. Both 0.2 mM Mn^{2+} and 5 mM Mg^{2+} were tested as metal ions for peak I and peak II DNA polymerase with poly(A)·oligo(dT), poly(dA)·oligo(dT), or poly(A)·poly(dT) as template. For each template with both enzymes, Mn^{2+} stimulated a greater rate of DNA synthesis than Mg^{2+} (data not shown). Several conclusions can be drawn from the results in Table III. (a) Only peak II DNA polymerase could effectively use poly(Cm)·oligo(dG) as template. (b) Peak I and IV DNA polymerase definitely prefered a synthetic template with regions of single-stranded DNA, the ratios of incorporation with poly(dA)·oligo(dT) *vs.* poly(A)·oligo(dT) being approximately 60:1 and 100:1, respectively. (c) Peak II and III enzyme prefered poly(A)·

TABLE III: The Relative Activity with Various Templates of Sephadex G-200 Purified DNA Polymerases from 1798 Cells.[a]

Template	Relative Rate of Incorporation			
	Peak I	Peak II	Peak III	Peak IV
1. Activated calf thymus DNA	0.290	0.039	0.500	0.093
2. Poly(dA)·oligo(dT)	1.00 (22.3)[b]	0.020	0.237	1.00 (10.2)
3. Poly(A)·oligo(dT)	0.017	1.00 (10.5)	1.00 (0.38)	0.009
4. Poly(A)·poly(dT)	0.020	0.920	0.840	0.083
5. Poly(C)·oligo(dG)	0.001	0.234	0.026	0.001
6. Poly(Cm)·oligo(dG)	0.003	0.250	0.026	0.001

[a] Aliquots from the peak fraction of each 1798 DNA polymerase from the Sephadex G-200 columns described in Figure 2 (peak I, fraction 57; peak II, fraction 74; peak III, fraction 60; and peak IV, fraction 86) were assayed for DNA polymerase activity with six different templates. All reaction mixtures (0.1 ml) contained 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, and either 12 μM [^{3}H]TTP (4700 cpm/pmol) or 9 μM [^{3}H]dGTP (1700 cpm/pmol). Other constituents in each type of reaction mixture were the following: (1) 50 μg/ml of activated calf thymus DNA, 5 mM $MgCl_2$ and 100 μM each of dATP, dCTP, and dGTP, (2) 58 μM poly(dA), 20 μM oligo(dT), and 0.2 mM $MnCl_2$, (3) 90 μM poly(A), 20 μM oligo(dT), and 0.2 mM $MnCl_2$, (4) 90 μM poly(A), 14 μM poly(dT), and 0.2 mM $MnCl_2$, (5) 52 μM poly(C), 13 μM oligo(dG), and 5 mM $MgCl_2$, and (6) 52 μM poly(Cm), 13 μM oligo(dG), and 0.2 mM $MnCl_2$. [b] The numbers in parentheses are the pmoles of [^{3}H]deoxyribonucleotide incorporated per 30 min per μl of enzyme with the best template for each enzyme.

oligo(dT) over poly(dA)·oligo(dT) as template, the ratios of incorporation being approximately 50:1 and 4:1, respectively.

Peak II DNA Polymerase is an RNA Directed DNA Polymerase. We have shown poly(Cm)·oligo(dG) to be an effective template for viral RNA directed DNA polymerase, and peak II DNA polymerase was the only enzyme found in 1798 cells which used poly(Cm)·oligo(dG) as template. The presence in extracts of nontransformed 3T6 cells of a DNA polymerase eluting from phosphocellulose at the same salt concentration as peak II enzyme could not be detected with either poly(A)·oligo(dT) or poly(Cm)·oligo(dG). Finally, peak II DNA polymerase eluted at the same salt concentration on phosphocellulose and appeared to have the same molecular weight as the DNA polymerase from H-MSV(MLV). Therefore, peak II DNA polymerase is probably of viral origin and appears to be identical with H-MSV(MLV) RNA directed DNA polymerase. To unequivocably establish that a DNA polymerase is a true "reverse transcriptase," the enzyme must be able to transcribe heteropolymeric regions of natural RNA (*e.g.*, viral 70S RNA) and the DNA product formed must be complementary to the template RNA (Rokutanda *et al.*, 1970; Spiegelman *et al.*, 1970a).

Peak II DNA polymerase was able to transcribe viral 70S RNA and the characteristics of the reaction are described in Table IV. With either AMV or M-MSV(MLV) 70S RNA, the reaction was sensitive to pretreatment of the RNA template with RNase, required Mn^{2+}, and required all four deoxyribonucleoside triphosphates, and all four labeled deoxyribonucleoside monophosphates were incorporated into DNA. The complementarity between the peak II DNA product and template RNA was demonstrated by two methods. Labeled DNA product was annealed to an excess of homologous or heterologous 70S RNA and the amount of DNA product in DNA–RNA hybrid form was determined (1) by resistance of the DNA to digestion by *A. oryzae* S_1 nuclease or *Neurospora crassa* nuclease, and (2) by Cs_2SO_4 equilibrium density centrifugation. Both S_1 and *Neurospora* nuclease specifically degrade single-stranded DNA while DNA in a RNA–DNA hybrid is resistant to digestion. The properties of these enzymes and their use to determine the amount of homology between DNA and RNA have been documented in detail (Leong *et al.*, 1972). Determination with nucleases of the extent of hybridization between AMV [^{3}H]DNA synthesized with AMV 70S RNA as template by peak II DNA polymerase and 70S RNA is presented in Table V. The results indicate that 60–90% of the AMV [^{3}H]DNA product was complementary to AMV 70S RNA. AMV [^{3}H]DNA annealed in the presence of RD-114 70S RNA was digested to background levels. Figure 3 shows the profiles on Cs_2SO_4 density gradients of [^{3}H]DNA synthesized with MSV 70S RNA as template after annealing to MSV 70S RNA (Figure 3A) and AMV 70S RNA (Figure 3B). The results indicate that at least 60% of the MSV [^{3}H]-DNA hybridized to MSV 70S RNA (the DNA banded at a density greater than 1.53 g/ml), while less than 5% hybridized to AMV 70S RNA.

Resistance of Poly(Cm) to Attack by Nuclease Activity Present in Murine Cultured Cells. We have shown that in the murine system, poly(Cm)·oligo(dG) with Mn^{2+} is a specific template for viral RNA-directed DNA polymerase. Poly(C)·oligo(dG) with Mg^{2+} has also been used to distinguish viral RNA-directed DNA polymerase from cellular DNA polymerases in cells of several different animal species (Bolden *et al.*, 1972; Weissbach *et al.*, 1972; Sarngadharan *et al.*, 1972). We found poly(C)·oligo(dG) to be specific for viral RNA-directed DNA polymerase in 1798 cells (see Table IV), and the enzyme (peak II) responded to poly(Cm)·oligo(dG) and poly(C)·oligo(dG) with about equal efficiency. In the analysis of crude cellular extracts for "reverse transcriptase" activity, however, poly(Cm)·oligo(dG) has a distinct advantage over poly(C)·oligo(dG). Poly(Cm) is resistant to attack by many nucleases which degrade poly(C), thus reducing the template efficiency of the polyribonucleotide. In a reaction mixture (0.1 ml) containing 50 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 0.2 mM $MnCl_2$, 1.4 mM poly(C) or 1.2 mM poly(Cm), and 50 μl of dialyzed 3T6 S-100 (see Materials and Methods), poly(Cm) was completely resistant to degradation during a 40-min incubation at 37°. In contrast, poly(C) was degraded at a linear rate during an equal time period, *i.e.*, 19, 40, 58, and 90% of the polymer was solubilized after 10, 20, 30, and 40 min, respectively, of incubation. The amount of polymer solubilized was determined by reading the absorbance at 270 nm of samples from reaction mixtures after precipitation of undegraded polymer with 0.01 M lanthanum nitrate in 0.1 M HCl. A similar time course for the degradation of [^{3}H]poly(C)

TABLE IV: The Characteristics of AMV and M-MSV(MLV) 70S RNA Directed DNA Synthesis by Peak II Enzyme.[a]

Additions	pmoles of [^{3}H]Deoxyribonucleotide Incorporated/90 min	
	AMV 70S RNA	M-MSV(MLV) 70S RNA
Complete ([^{3}H]TTP)	0.34	0.53
Plus RNase (20 μg/ml)	<0.01	0.04
Minus 70S RNA	0.02	0.03
Minus Mg^{2+}	1.01	0.63
Minus Mn^{2+}	0.01	0.01
Minus Mg^{2+} and Mn^{2+}	<0.01	0.03
Minus dATP	0.04	
Minus dATP, dCTP, dGTP	0.02	0.08
Complete ([^{3}H]dATP)	0.31	0.35
Complete ([^{3}H]dCTP)	0.12	0.20
Complete ([^{3}H]dGTP)	0.16	0.46

[a] The peak fraction of peak II enzyme from a Sephadex G-200 column identical with that described in Figure 2A (9 mg of protein was applied to this column) was assayed in a reaction mixture (0.1 ml) containing 20 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 30 mM NaCl, 30 μl of Sephadex G-200 purified peak II, and the constituents indicated above. The concentration of unlabeled deoxyribonucleoside triphosphates was 100 μM; $MnCl_2$ and $MgCl_2$ were both present in the complete reaction mixture at 0.2 and 5 mM, respectively. When RNase A was added, the complete reaction mixture was preincubated for 1 hr at 37° in the absence of peak II DNA polymerase. AMV and M-MSV(MLV) 70S RNA were at 19 and 9 μg/ml, respectively. The concentrations of the labeled deoxyribonucleoside triphosphates were: 15 μM [^{3}H]TTP (27,000 cpm/pmol), 12 μM [^{3}H]dATP (4700 cpm/pmol), 8 μM [^{3}H]dCTP (14,000 cpm/pmol), and 18 μM [^{3}H]-dGTP (1800 cpm/pmol).

TABLE V: Demonstration of the Complementarity between [^{3}H]DNA Synthesized from AMV 70S RNA by Sephadex G-200 Purified Peak II Enzyme and Viral 70S RNA. Determination by the Use of S_1 and *Neurospora* Nuclease.[a]

[^{3}H]DNA Annealed with 70S RNA From	Nuclease Added		Cl_3CCOOH Insoluble cpm	% Resistant to Nuclease
	S_1	*Neurospora*		
AMV	−		1120	
	+		967	87
AMV		−	1083	
		+	661	61
RD-114	−		1096	
	+		76	7
RD-114		−	1737	
		+	171	10
None	−		1085	
	+		85	7
None		−	1212	
		+	74	6

[a] AMV [^{3}H]DNA prepared and hybridized to viral 70S RNA as described in Materials and Methods was treated with S_1 and *Neurospora* nuclease to determine the extent of hybridization. A 50-μl aliquot from each hybridization mixture was divided equally into two reaction mixtures each containing 0.03 M sodium acetate (pH 4.5), 0.3 M NaCl, 1.8 mM $ZnCl_2$, and 10 μg/ml of denatured calf thymus DNA in a final volume of 1.5 ml. S_1 nuclease, 200 μl, was added to only one of each duplicate reaction mixture. After a 2-hr incubation at 37°, 50 μg of carrier calf thymus DNA was added, and the [^{3}H]DNA insoluble in 10% Cl_3CCOOH was determined. For treatment with *Neurospora* nuclease, a dialyzed sample (Materials and Methods) from each hybridization mixture was divided equally into two reaction mixtures (0.1 ml) each containing 0.1 M Tris-HCl (pH 8.0), 0.01 M $MgCl_2$, and 10 μg/ml of denatured *E. coli* DNA. One reaction mixture received one unit of *Neurospora* nuclease. After 30 min at 37°, 100 μg of carrier calf thymus DNA was added, and the [^{3}H]DNA insoluble in 10% Cl_3CCOOH was determined.

by nuclease activity present in crude extracts of 3T6 cells was obtained by determining trichloroacetic acid soluble radioactivity. The advantage of using nuclease-resistant poly(2′-*O*-methylribonucleotides) for assay of enzyme activities in crude bacterial systems has also been demonstrated (Dunlap *et al.*, 1971).

Discussion

Based on template specificity, elution properties from phosphocellulose, and molecular weight, the DNA polymerases we have isolated from MSV(MLV) transformed mouse 3T6 cells (peaks I, III, and IV) can be compared with other enzymes already reported to be present in animal cells. DNA polymerases with analogous properties have been isolated from a number of different animal cell species. Peak I DNA polymerase(s) is similar to the high molecular weight enzyme which has been isolated from the cytoplasm of a variety of mammalian cell species (Yoneda and Bollum, 1965; Weissbach *et al.*, 1971; Baril *et al.*, 1971; Sedwick *et al.*, 1972). Peak III DNA polymerase is analogous to the R-DNA polymerase (Fridlender *et al.*, 1972; Bolden *et al.*, 1972) or DNA polymerase A (McCaffrey *et al.*, 1973) reported to be present in a number of animal cells. Peak IV DNA polymerase had some of the properties of the low molecular weight nuclear enzyme also present in mammalian cells (Weissbach *et al.*, 1971; Schlabach *et al.*, 1971; Chang and Bollum, 1971, 1972; Smith and Gallo, 1972). We emphasize that these identifications are only tentative. No attempt has been made to localize the enzyme activities in the cell, to determine the optimal assay conditions for each enzyme, or to determine the sensitivity of the enzymes to specific inhibitors. For the purposes of this report, it was sufficient to isolate enzymes representative of the different types of DNA polymerases reported to be present in normal mammalian cells, and to show that none of these DNA polymerases responded to poly(Cm)·oligo(dG) as template.

Peak II DNA polymerase from 1798 cells has many of the properties of viral RNA directed DNA polymerase, and appears to be identical with H-MSV(MLV) DNA polymerase. The molecular weight of 85,000 determined by gel filtration for peak II and H-MSV(MLV) DNA polymerase is in fairly good agreement with the 90,000 (Hurwitz and Leis, 1972) and 70,000 (Tronick *et al.*, 1972) reported for R-MLV and the 70,000 molecular weight reported for MSV(MLV) (Ross *et al.*,

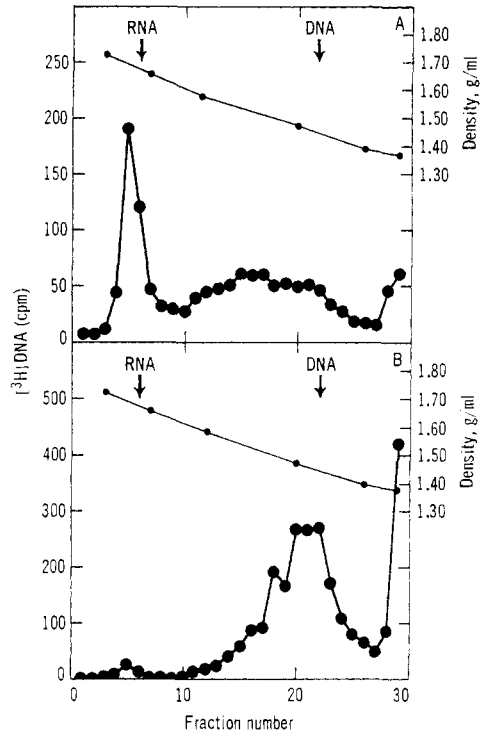

FIGURE 3: Demonstration of the complementarity between DNA synthesized by Sephadex G-200 purified peak II enzyme and template viral 70S RNA: determination by the use of Cs_2SO_4 equilibrium density gradient centrifugation of M-MSV(MLV)[^{3}H]DNA after annealing to M-MSV(MLV) 70S RNA (A) and to AMV 70S RNA (B). Viral 70S RNA at 200 μg/ml was incubated at 80° for 2 min with poly(U) at 2000 μg/ml. [^{3}H]DNA (400 pg) prepared as described in Materials and Methods was then added, and the hybridization mixture was brought to 3 × SSC, 0.01 M sodium *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (pH 7.4), 0.1% sodium dodecyl sulfate, 0.05 M EDTA, 45% formamide, 100 μg/ml of poly(U), and 10 μg/ml of 70S RNA in a volume of 500 μl. Incubation was at 37° for 24 hr. The hydridization mixtures were then dialyzed for 6 hr against two 1-l. changes of 0.1 × SSC, 100 μl of *E. coli* DNA (500 μg/ml) and 4.1 ml of saturated Cs_2SO_4 were added, the density was adjusted to 1.54 g/ml with water, and the mixture was centrifuged at 42,000 rpm in a 50 Ti rotor for 60 hr at 20°. Fractions were collected from the bottom of the tubes and the refractive index of appropriate fractions was determined. Calf thymus DNA (100 μg) was then added and the Cl_3CCOOH precipitable radioactivity was determined. [^{3}H]Adenovirus DNA and KB cell ribosomal [^{3}H]RNA were run in a separate tube as density markers.

1971). Peak II DNA polymerase was able to transcribe heteropolymeric regions of 70S RNA into complementary DNA, a catalytic activity thus far shown to be uniquely characteristic of viral RNA directed DNA polymerase.

The template specificity of the DNA polymerase from a putative human RNA tumor virus is not known. The chances that poly(Cm)·oligo(dG) would be an effective template for this enzyme seem quite good, since the DNA polymerase from every RNA tumor virus tested responded to poly(Cm)·oligo(dG). These include viruses of murine, feline, avian, and primate origin.

The specificity of poly(Cm)·oligo(dG) as template for RNA directed DNA polymerase in other tissue culture cell lines and the potential of the polymer for detecting "reverse transcriptase" activity in human neoplastic tissues are being evaluated. The results thus far obtained indicate that poly(Cm) complexed to a oligo(dG) primer can be used as a specific template for viral RNA directed DNA polymerase.

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Hydroxylapatite-Catalyzed Degradation of Ribonucleic Acid†

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ABSTRACT: Hydroxylapatite was found to catalyze the degradation of RNA at elevated temperatures. Single-stranded RNA was degraded considerably more rapidly than double-stranded RNA. DNA was not affected. Factors which decreased the adsorption strength of the RNA decreased the rate of degradation. Thus increasing the phosphate concentration of the surrounding buffer or substituting Cs^+ for Na^+ reduced the rate of hydroxylapatite degradation. The most likely mechanism appears to be a Ca^{2+} ion mediated hydrolysis which occurs primarily within a few angstroms of the crystal surface where the free Ca^{2+} ion concentration is several orders of magnitude higher than in the bulk solution. Caution is advised in the design and interpretation of experiments involving the chromatography of RNA on hydroxylapatite. However, the degradation of RNA by hydroxylapatite together with the ability of hydroxylapatite to fractionate nucleic acids according to size can be used for the controlled fragmentation of RNA preparations to preselected molecular weights in a simple one-step procedure.

Hydroxylapatite is becoming ever more widely used in the analysis of nucleic acids (Bernardi, 1971; Kohne and Britten, 1971; Muench, 1971). Much work now being done involves the use of elevated temperatures (Kohne and Britten, 1971) and many experiments are done which involve RNA. We have found previously that single-stranded RNA (ss-RNA)[1] but not DNA is degraded rapidly by hydroxylapatite in dilute buffer at high temperatures (Martinson, 1973c). We have now studied this phenomenon in greater detail in order first to try and elucidate the mechanism involved, second to assess its impact on the interpretation of conventional experiments, and third to explore the possibility of using the system as a convenient way to produce RNA fragments of defined size. It was found that adsorption on the hydroxylapatite surface is required for enhanced degradation to occur. Furthermore rigid ds-RNA is degraded much more slowly than ss-RNA. Consequently the extent of RNA cleavage is less for ds-RNA and probably also RNA–DNA hybrids than it is for ss-RNA during high-temperature chromatography on hydroxylapatite. Because the affinity of nucleic acids for hydroxylapatite decreases with molecular weight (Martinson, 1973c; Piperno *et al.*, 1972), degraded RNA fragments are released from the crystal surface when they become small enough. This limit size can be controlled by the eluting power of the buffer in which incubation is conducted and the system can therefore be adapted for the production of ss-RNA fragments of defined size.

Materials and Methods

The viral RNAs were gifts of Dr. L. Lewandowsky and Dr. C. A. Knight.

Hydroxylapatite chromatography was conducted as described previously (Martinson, 1973a,b) using either the Bio-Rad HT or HTP. Where indicated the hydroxylapatite was preincubated in 0.1 M phosphate in a boiling water bath for 30 min.

Linear sucrose gradients (0.25–0.65 M) which were 10 mM in Na_3EDTA and also either 10 mM in Tris-phosphate (pH 7.2) or 0.2 M in NaCl were run in the Beckman SW 41 rotor at 41,000 rpm. Fractions were collected by puncturing the bottom of the tube with a hypodermic needle and collecting a specified number of drops per fraction.

Polyacrylamide gel (5%) electrophoresis (Loening, 1967) was conducted at room temperature in 0.2% sodium dodecyl sulfate for 2 or 3 hr. Dr. G. Schultz kindly provided 4s and 5s size standards and Dr. R. B. Church provided the facilities.

Ribonuclease sensitivity was determined by adding 0.2 ml

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[1] Abbreviations used are: ds-RNA, double-stranded RNA; ss-RNA, single-stranded RNA.