JOURNAL OF VIROLOGY, Mar. 1974, p. 712-720

Vol. 13, No. 3
Printed in U.S.A.

# Characterization of an Endogenous RNA-Dependent DNA Polymerase Associated with Murine Intracisternal A Particles

STRINGNER SUE YANG AND NELSON A. WIVEL

*Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014*

Received for publication 2 November 1973

An RNA-dependent DNA polymerase associated with intracisternal A particles has been characterized. The enzyme required $Mg^{2+}$ or $Mn^{2+}$, dithiothreitol and the presence of all four deoxyribonucleoside triphosphates for the expression of maximal activity. Sensitivity of the endogenous RNA-dependent DNA polymerase activity to a low concentration of pancreatic ribonuclease in the presence of a high concentration of NaCl suggested that the enzyme might be utilizing the A particle endogenous RNA as template. Evidence in support of this was provided by analyses of early and late DNA products of the endogenous reaction by $Cs_2SO_4$ isopycnic gradient centrifugation and hybridization of purified 60 to 70*S* and 35*S* RNAs of A particles with the purified DNA product.

Recent reports strongly suggest that intracisternal A particles represent some expression of a viral genome, since they contain a high molecular weight, 60 to 70*S* RNA as the endogenous nucleic acid, and a group-specific structural protein of 70,000 molecular weight (14, 35).

Their occurrence has been frequently observed in tumors of the gerbil (29), rat (19), guinea pig (18), man (25), and in both normal and neoplastic mouse tissues (6-8, 33). Such particles are most probably vertically transmitted from oocyte to the fertilized embryo, a phenomenon analogous to murine C-type viruses, and their transient bursts of proliferation during early embryonic development have been described (5).

In mice they are most abundant in certain tumors and can be obtained in significant quantities from such neoplasms as the plasma cell tumor, squamous carcinoma, and neuroblastoma. Advances in the biochemical studies of intracisternal A particles have been difficult because of problems involving cellular contamination inherent in their isolation from cytoplasmic cisternae. The cryptic expression or the loss of biochemical function, as a result of strenuous purification procedures, must be considered. An earlier report described the existence of a novel DNA polymerase activity associated with intracisternal A particles that lacked endogenous activity, that did not require detergent activation, and that was solely dependent on the synthetic template oligo $dT_{12-18}$·poly rA. It has been referred to as a poly (dT) polymerase (32). In this communication definitive evidence is presented to demonstrate the existence of an endogenous RNA-dependent DNA polymerase associated with intracisternal A particles. This polymerase utilizes its own endogenous RNA as template; it synthesizes a DNA product that is in the form of an RNA-DNA hybrid at the end of a short-term reaction, and primarily DNA at the end of a long-term reaction. The DNA product hybridizes back to A particle RNA.

## MATERIALS AND METHODS

**Tumor and cells.** A mouse plasma cell tumor (MOPC-104E) was kindly supplied by M. Potter of the National Cancer Institute, and was maintained by subcutaneous transplantation in BALB/c mice in this laboratory. Neuroblastoma (N-18), a continuous tumor cell line (35), was cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 units/ml), and streptomycin (25 $\mu$g/ml) in a 5% $CO_2$ atmosphere at 37 C. Cells were fed on alternate days. All tissue culture materials were from GIBCO.

**Isolation and purification of intracisternal A particles.** Preparation of purified intracisternal A particles has been previously described (15, 35). Briefly, cytoplasmic extracts from tumor homogenates were centrifuged at 13,000 × *g* for 20 min to obtain a pellet of mitochondria and microsomes. A particles were freed from microsomal vesicles through the use of Triton X-100 and mechanical shearing. The freed particles were further purified through three centrifugation cycles in discontinuous sucrose (25 to 48%) gradients. Finally, A particles were subjected to isopycnic centrifugation in linear gradients utilizing 33 to 68% (wt/vol) sucrose or 35 to 85% glycerol, and banded at 1.21 to 1.23 g/cm$^3$. The appearance of a representative preparation is depicted in Fig. 1. It

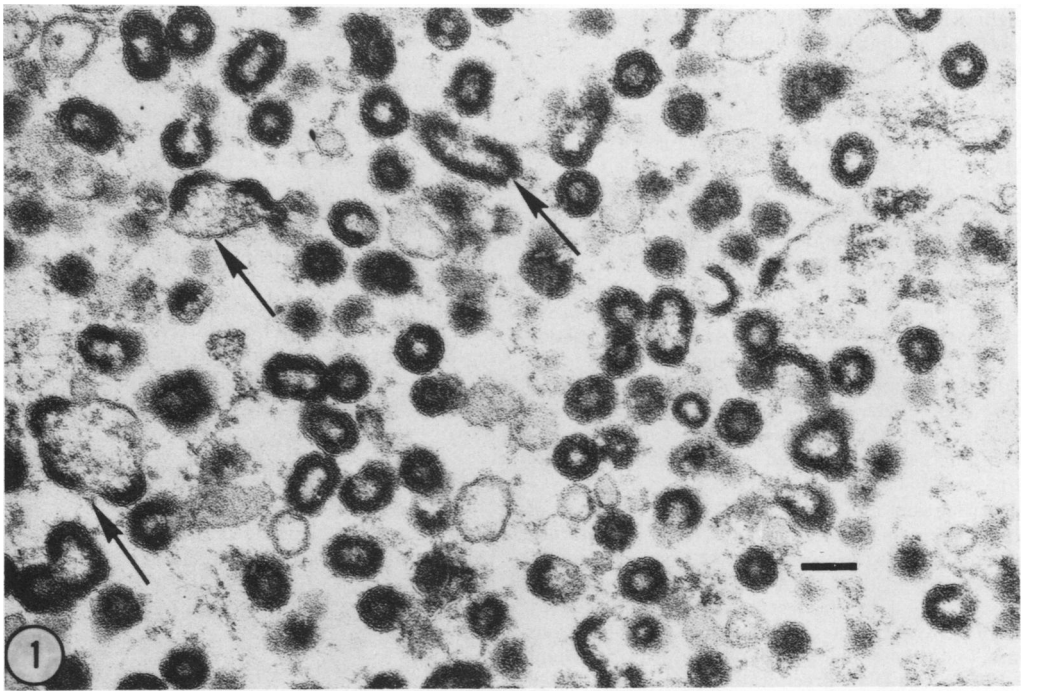

FIG. 1. *Thin section preparation of gradient purified intracisternal A particles. In addition to complete single particles there are numerous elongated forms made up of multiple buds (arrows). ×70,000. The bar represents 0.1 μm.*

should be emphasized that N-18 neuroblastoma cells showed no C-type viruses as examined ultrastructurally, nor did the A particle isolates contain C-type viral antigens (35). Although the whole cells from MOPC-104E plasma cell tumor contain C-type viral antigens, the purified A particles do not (35). Furthermore, the purification scheme rules out the chances for contamination by C-type viruses as demonstrated by a reconstitution experiment in which Rauscher leukemia virus (RLV) labeled with $^3$H-uridine was added to the initial tumor cell suspension prior to the isolation and purification of intracisternal A particles. All RLV-associated tritium counts were removed at the initial discontinuous sucrose gradient centrifugation (35). A particles purified by a final linear gradient centrifugation step therefore exhibit biochemical properties that can be appropriately considered as intrinsic to A particles alone.

**DNA polymerase assays.** Details of DNA polymerase assay have been described earlier (2, 28, 34) except for some minor variations used to optimize the assay conditions. Briefly, in a final volume of 50 μliters, the assay mixture contained Tris-hydrochloride, pH 8.1, 50 mM; Mg acetate, 10 mM, or $MnCl_2$, 0.8 mM; dithiothreitol (DTT), 8 mM; KCl, 75 mM; and approximately 5 μg of purified intracisternal A particles treated with 0.018% Triton X-100 or X-102 for 10 min at 0 C. For assaying the endogenous reaction, cold dCTP, dGTP, and dATP at a final concentration of $8 \times 10^{-5}$ M were added along with 5 μCi of $^3$H-TTP, and the reaction was carried out at 37 C. For assays with synthetic templates, a template concentration of 50 μg/ml was used along with the appropriate substrates at 30 C. The reaction was terminated by additions of 2 ml of ice-cold 0.08 M sodium pyrophosphate in 12.5% trichloroacetic acid and 20 μg of yeast nucleic acid as carrier. The radioactive precipitates were then collected on Millipore filters, washed extensively with 5% trichloroacetic acid, washed with 80% ethanol, dried, and then counted in Liquifluor by a liquid scintillation method.

All chemicals, unless specified, were obtained from Calbiochem, Sigma, and Fisher Scientific. Triton X-102 and X-100 were obtained from Sigma and from Packard Instrument Co., respectively. All isotopes were purchased from New England Nuclear Corp. Bovine pancreatic RNase and DNase were obtained from Worthington Biochemicals. RNase at 0.25 mg/ml was boiled for 10 min to inactivate the contaminant DNase prior to use.

**Virus purification.** Avian myeloblastosis virus (AMV) was kindly supplied by J. W. Beard and D. P. Bolognesi through the Special Virus Cancer Program of the National Cancer Institute. Further purification of AMV was carried out by the methods described earlier (4). AMV materials banding at a density of 1.16 g/cm$^3$ were used for RNA extraction. Highly purified RLV ($10^{11}$ particles/ml) was obtained from Electronucleonics Inc. and subjected to isopycnic centrifugation in a 35 to 85% glycerol gradient as described. Materials banding at the 1.16 g/cm$^3$ area and coincident with reverse transcriptase activity were used for RNA extraction.

**RNA extraction and purification.** High molecular

weight RNA was extracted from purified AMV and from RLV, and purified by velocity gradient centrifugation prior to being used as templates in DNA polymerase assays. Details of the extraction and purification procedures have been documented elsewhere (35). The majority of the viral RNA from either AMV or RLV, when purified to homogeneity, sedimented at the 60 to 70*S* area. This fraction was concentrated by precipitation in ethanol-NaCl and the RNA was resuspended in 0.1 M NaCl and 5 mM $MgCl_2$ prior to use as templates.

**Templates.** Natural templates, unless specified, were purchased from Miles laboratory. Salmon sperm DNA was activated with a limited DNAase digestion at 37 C for 15 min as described elsewhere (21), followed by extensive purification as described below for DNA products. Copolymers and homopolymers of various synthetic oligonucleotides with specified chain lengths were obtained from either Miles Laboratory or Collaborative Research Corp.

**Purification and analysis of DNA products of the endogenous RNA-dependent DNA polymerase reaction.** The radioactive products from the endogenous RNA-dependent DNA polymerase reaction were adjusted to 0.3 M Na acetate, pH 5.0, purified by phenol-SDS extraction twice, ether extraction four times, and then ethanol-NaCl precipitation overnight at −20 C. The DNA precipitates were then collected by centrifugation, redissolved in 0.3 M Na acetate, and applied to a sulfopropyl Sephadex C-50 column. Fractions eluted at the void volume were pooled and reprecipitated in ethanol-NaCl prior to $Cs_2SO_4$ isopycnic centrifugation analysis at 40,000 rpm for 60 h in the SW 50 rotor. Gradient samples were weighed for density determination prior to precipitation in 12% trichloroacetic acid and processed for radioactive determination.

**Hybridization reactions and analysis of the RNA-DNA hybrids with S-1 exonuclease.** $^3$H-DNA product of the endogenous RNA-dependent DNA polymerase of intracisternal A particles was prepared in the presence of 5 μg of actinomycin D per ml as described in the previous sections. The $^3$H-DNA was passed through a Sephadex G50 column (180 by 0.8 cm) instead of an SP Sephadex C50 column. The $^3$H-DNA recovered at the void volume was adjusted to 0.3 N KOH and was hydrolyzed at 100 C for 10 min to remove any RNA. The sample was then neutralized with HCl and adjusted to 3 × SSC (1 × SSC-0.15 M NaCl-0.015 M trisodium citrate) and 50% formamide (Eastman-Kodak) for hybridization. Purified intracisternal A particle 60 to 70*S* RNA and 35*S* RNA at about 2.0 μg was then added to 200 μliters of the hybridization mixture, and the reaction was carried out at 37 C for 18 h. Control samples were prepared in the same manner without or with heterologous RNA, such as Qβ RNA, at the same concentration. At the end of the hybridization reaction, the samples were adjusted to a final concentration of 25 mM Na acetate (pH 4.5), 0.5 mM $ZnSO_4$, 0.15 M NaCl, and 20 μliters of purified S-1 exonuclease. The nuclease assay was then continued at 45 C for 90 min. At the end of the assay, the reaction was terminated with precipitation in 12% trichloroacetic acid with 20 μg of yeast transfer RNA (Miles Laboratory) as carrier. The radioactivity was determined as described earlier.

**Preparation of S-1 exonuclease.** S-1 exonuclease was purified from crude α-amylase powder (Sigma Chemical Co.) from *Aspergillus oryzae* according to the procedures described by Sutton (26). It has a specific activity of hydrolyzing about 1,000 counts/min of $^3$H-DNA (single stranded) per 60 min per 2 μliters of the enzyme preparation at 45 C.

**Protein determination.** Protein was determined by the Lowry method (16). Since glycerol, sucrose, and Triton all interfere with the colorimetric determination, samples were first precipitated with 10% perchloric acid (PCA) and washed three times with PCA prior to alkali digestion; and then the protein determination was carried out.

## RESULTS

**Association of an endogenous RNA-dependent DNA polymerase with intracisternal A particles.** Figure 2 demonstrates the association of an endogenous RNA-dependent DNA polymerase activity with purified intracisternal A particles which sedimented at the density of 1.21 to 1.23 g/cm$^3$ across a linear sucrose gradient. When each fraction of the gradient was assayed under optimal conditions, as described in the next section, the endogenous RNA-dependent DNA polymerase activity peaked sharply and uniformly at 1.21 to 1.23 g/cm$^3$. It should be noted that there were no other contaminating materials such as C-type particles since no polymerase activity was observed at

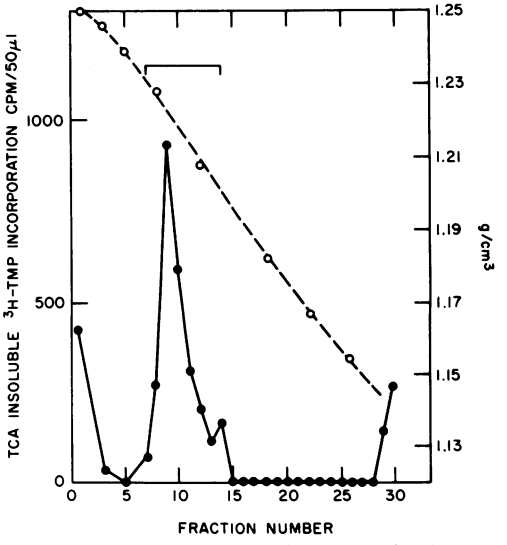


FIG. 2. *Linear sucrose gradient analysis of an endogenous RNA-dependent DNA polymerase activity associated with murine intracisternal A particles. All experimental procedures were described in Materials and Methods.*

1.16 g/cm$^3$. This was confirmed by the electron-microscopy presented in Fig. 1. The possibility of contaminating C-type particles that might confuse the biochemical studies of intracisternal A particles was ruled out by the immunological studies and the reconstitution experiment reported earlier (35). The endogenous RNA-dependent DNA polymerase activity that sedimented at the 1.21 to 1.23 g/cm$^3$ area can therefore be regarded as intrinsic to intracisternal A particles especially when the following evidence is considered.

**Characteristics of an endogenous RNA-dependent DNA polymerase associated with intracisternal A particles.** Table 1 summarizes the conditions essential for optimal activity of the endogenous DNA polymerase associated with intracisternal A particles. Similar to the characteristics of other DNA polymerases, the requirements for a complete endogenous reaction include DTT, divalent cation, $Mg^{2+}$ or $Mn^{2+}$ (see Fig. 4), monovalent cation, $Na^+$ or $K^+$, and the presence of all four deoxyribonucleoside triphosphates. The recovery of the endogenous DNA polymerase reaction in the absence of $Na^+$ or $K^+$ was 62 to 64% probably due to the significant amount of $Na^+$ ion present in all four deoxyribonucleoside triphosphates. Total depression of activity was achieved by omitting three deoxyribonucleoside triphosphates from the reaction. Among the four DNA precursors, dATP seems to be the most essential, since its deletion led to a reduction of 80% of the activity. This characteristic of the endogenous reaction of intracisternal A particles DNA polymerase resembles that reported for the reverse transcriptase of Rous sarcoma virus (28) and Mason-Pfizer monkey virus (1).

**Detergent activation.** This endogenous DNA polymerase activity is dependent on detergent activation to some degree. Without preincubation of the particles with Triton X-102, only 26% of the control activity was observed (Table 1), a phenomenon reminiscent of reverse transcriptase of murine sarcoma viruses (13). Clearly, this suggests that detergent activation is required either for solubilizing the DNA polymerase for reactivity or to provide accessibility of the substrates, or both. At this point, it should be clarified that the Triton X-100 used in the isolation procedure is essential to free the A particles from microsomal vesicles. At the chosen concentration (0.2%), the function of the detergent is not to lyse the microsomal vesicles, but to sensitize the membranes to disruption by mechanical shear. This allows for the release of completely formed A particles which are morphologically intact and still exhibit significant intrinsic biochemical properties such as 60 to 70*S* RNA (35). However, there are many budding forms, and shearing them free of the membranes leaves open-ended particles (Fig. 1); these incomplete particles are obviously susceptible to leakage of intrinsic RNA and DNA polymerase as a result of the isolation procedure. It has to be considered that the DNA polymerase activity being assayed is that primarily derived from the population of completely formed intracisternal A particles.

TABLE 1. *Characteristics of endogenous RNA-dependent DNA polymerase activity associated with intracisternal A particles*[a]

| Conditions | Acid-insoluble $^3$H-TMP incorporation[b] | Control (%) |
|---|---|---|
| Complete | 13.34 | 100 |
| Minus $Mg^{2+}$ | 0.38 | 3 |
| Minus DTT | 0.27 | 2 |
| Minus $Na^+$ | 8.61 | 64 |
| Minus $K^+$ | 8.20 | 62 |
| Minus dATP[c] | 2.65 | 20 |
| Minus dGTP[c] | 6.20 | 47 |
| Minus dCTP[c] | 8.30 | 63 |
| Minus dA, G, C, TP | 0.64 | 5 |
| Minus Triton X-102 treatment | 3.50 | 26 |
| Preincubation with RNase | 0.35 | 3 |

[a] All assay conditions are described in the text. Complete condition refers to inclusion of all critical requirements such as $K^+$ or $Na^+$, $Mg^{2+}$, DTT, and all four deoxyribonucleoside triphosphates, and 0.018% Triton X-102 treatment. Each assay contained 24 μg of A particle protein (MOPC-104E plasma cell tumor). Preincubation with RNase (25 μg/ml) was carried out in 0.15 M NaCl at 37 C for 2 h; 12,970 counts/min = 1.0 pmol.

[b] Measured in picomoles per hour per milligram of protein.

[c] Acquired from Sigma Chemical Co. in the form of disodium salt.

**RNase sensitivity.** RNase digestion experiments indicated that indeed the endogenous DNA polymerase of A particles most probably was dependent on the intrinsic RNA as template (Table 1 and Fig. 3). RNase at low concentration, 25 μg/ml, but in the presence of high salt, 0.15 M NaCl, for the most part digests only single-stranded RNA (20). Under these conditions, intracisternal A particles previously treated with Triton X-102 and incubated at 37 C for 2 h lost all endogenous RNA-dependent DNA polymerase activity (Fig. 3D). Under the same conditions but using an even lower RNase

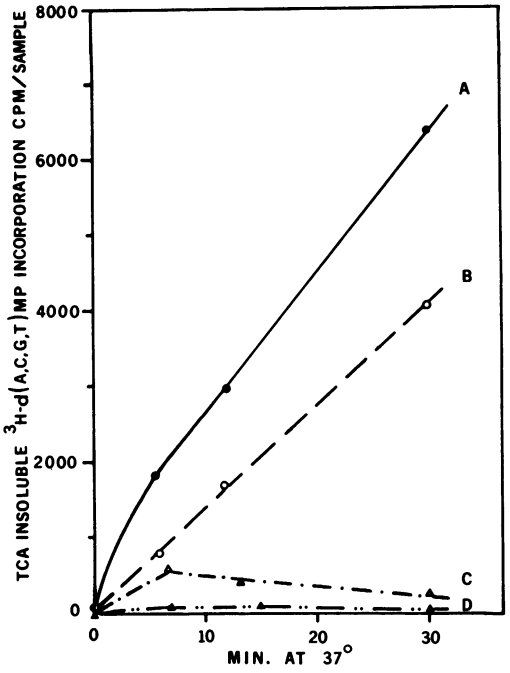


FIG. 3. *Sensitivity of the endogenous RNA-dependent DNA polymerase activity of intracisternal A particles to pancreatic ribonuclease digestion at high NaCl concentration. Approximately 125 μg of intracisternal A particles were preincubated with Triton X-102 at a final concentration of 0.018% in the presence of 0.15 M NaCl. A, At 0 C for 2 h, ●; B, at 37 C for 2 h, ○; C, at 37 C with 16.6 μg of pancreatic RNase per ml for 1 h, △; and D, at 37 C with 25.0 μg of pancreatic RNase per ml for 2 h prior to polymerase assays, ▲. Each sample was adjusted to complete assay conditions to a final volume of 250 μliters with 5 μCi each of all four $^3$H-deoxyribonucleotide triphosphates (10 Ci/mmol). Samples (50 μliters each) were withdrawn at specified times, precipitated in 12% trichloroacetic acid-pyrophosphate and processed as described in the text.*

concentration, 16.6 μg/ml, and a shorter period of incubation, 60 min, A particles still lost about 87% of their endogenous activity at the end of a 30-min reaction (Fig. 3C). Further proof of the involvement of intrinsic RNA in the endogenous DNA polymerase reaction of intracisternal A particles is evident when the nature of the DNA product is considered below.

**Kinetics of the RNA-dependent DNA polymerase reaction.** Under optimal conditions, the rate of incorporation of acid-insoluble $^3$H-TMP in an endogenous reaction by detergent treated intracisternal A particles varied among preparations. Figure 4A-1 depicts the kinetics of a more active preparation (number 1) in which the endogenous reaction is linear up to 30 to 40 min at 37 C before it slowed. However, A particle preparations varied in the extent of their endogenous reaction even at the same protein concentration (Fig. 4A-1 versus 4A-2). This was probably due to the availability of the RNA template; leakage of RNA from A particles due to isolation procedure could account for the paucity of an endogenous reaction in one preparation of A particles as compared with another. A particles isolated and purified from frozen tumor tissues frequently exhibited much lower endogenous activity (Fig. 4B-3), but given the presence of the synthetic hybrid template, $dT_{12\text{-}18}$·poly rA, the initial rate of the RNA-dependent DNA polymerase activity was significantly increased (Fig. 4B-1 and 2). The kinetics of the polymerase reaction in the presence of the $dT_{12\text{-}18}$·poly dA, the DNA-DNA template, were much slower (Fig. 4B-4). This seems reasonable since $dT_{12\text{-}18}$·poly dA did not stimulate the intracisternal A particle DNA polymerase.

**Template preference.** A variety of natural RNAs and DNAs, including activated salmon sperm DNA, $T_2$ phage DNA, AMV 70S RNA, RLV 70S RNA, MS-2 RNA, vesicular stomatitis

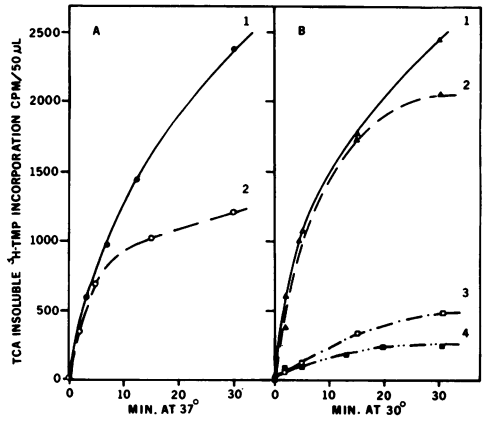


FIG. 4. *Kinetics of the RNA-dependent DNA polymerase of intracisternal A particles with and without exogenous synthetic templates. All assay conditions were described in the text. In A, the amount of purified A particles from MOPC-104E plasma cell tumor used in the assay depicted by curve number 1 (●) was 15 μg, whereas 12.5 μg of purified A particles from N-18 neuroblastoma cells was used in the assay depicted by curve number 2 (○). In B, 12.5 μg of another purified preparation of A particles isolated from previously frozen MOPC-104E plasma cell tumor tissues was used in each kinetic study. Kinetics of A particle DNA polymerase in the presence of $dT_{12\text{-}18}$·poly rA was depicted by curve number 1 (▲), at 50 μg/ml, and by curve number 2 (△), at 25 μg/ml. Curve number 3 (□) depicted kinetics of the endogenous activity of the same A particles. Curve number 4 (■) depicted kinetics of A particle DNA polymerase with $dT_{12\text{-}18}$·poly dA, 50 μg/ml as template.*

virus RNA, influenza virus RNA, and Qβ RNA, were used to assess template preference. In all cases the copying of these nucleic acids was weak or nonexistent.

Table 2 summarizes the effects of various synthetic templates on the RNA-dependent DNA polymerase activity of intracisternal A particles. At high concentration, 100 μg/ml, $dT_{12-18}$·poly rA is the most effective template, among all the synthetic oligonucleotides used in this study, stimulating the A particle DNA polymerase to 9.4-fold greater activity. At the same concentration, $dT_{12-18}$·poly dA did not stimulate the DNA polymerase activity. When these activities under the stimulation by the two templates are compared, a ratio ($dT_{12-18}$·poly rA/$dT_{12-18}$·poly dA) of 9.9 was observed, which is characteristic of viral reverse transcriptase (20). The same preferential ratio, although somewhat lower at 8.3, was observed when the template concentration was 50 μg/ml. Poly dT·poly rA, a template that stimulated both cellular and viral DNA polymerases (10, 20, 21), also stimulated the activity of A particle DNA polymerase. Both the DNA copolymer, poly d(A-T), and the RNA-RNA duplex, poly rA·poly rU, failed to stimulate A particle DNA polymerase to a higher activity. In all these respects, the DNA polymerase of intracisternal A particles and the poly dT polymerase (32) are similar.

Interestingly, the RNA-DNA hybrid template, $dG_{12-18}$·poly rC, that was reported as specific for viral reverse transcriptase (1, 20, 22) also stimulated the DNA polymerase of intracisternal A particles to about 300% of the control activity. Much greater stimulation was observed with other A particle preparations by this template. Poly dT polymerase was also reported to be stimulated by $dG_{12-18}$·poly rC (32). Similar to other C-type viral reverse transcriptases (1), intracisternal A particle DNA polymerase is not stimulated by $dC_{12-18}$·poly rG.

**Divalent cation requirement.** The endogenous RNA-dependent DNA polymerase activity was preferentially activated by $Mn^{2+}$ rather than by $Mg^{2+}$. Figure 5 shows a divalent cation concentration dependence of the endogenous DNA polymerase activity of A particles. Optimal $Mg^{2+}$ concentration varied from about 5.5 to 10 mM; however, the extent of the DNA polymerase activity at optimal $Mg^{2+}$ concentration was much less than that observed at optimal concentration of $Mn^{2+}$. The optimal concentration for $Mn^{2+}$ was rather sharp and was found to be at 0.8 mM for the activation of the endogenous RNA-dependent DNA polymer-

TABLE 2. *Comparison of stimulatory effects of various synthetic templates on DNA polymerase activity of intracisternal A particles*

| Templates | Incorporation of acid-insoluble deoxyribonucleotide[a] | Control (%) |
|---|---|---|
| None—endogenous (μg/ml) | 0.17[b] | 100 |
| $dT_{12-18}$·poly rA (100) | 1.59[b] | 935 |
| $dT_{12-18}$·poly dA (100) | 0.16[b] | 94 |
| $dT_{12-18}$·poly rA (50) | 1.20[b] | 706 |
| $dT_{12-18}$·poly dA (50) | 0.14[b] | 83 |
| poly dT·poly rA (50) | 0.69[b] | 406 |
| poly d(A-T) (50) | 0.15[b] | 90 |
| poly rA·poly rU (50) | 0.11[b] | 65 |
| None—endogenous | 0.14[c] | 100 |
| $dC_{12-18}$·poly rG (50) | 0.17[c] | 125 |
| None—endogenous | 0.16[d] | 100 |
| $dG_{12-18}$·poly rC (50) | 0.48[d] | 300 |

[a] Measured in picomoles per 50 μliters per 30 min. All assay conditions are described in Materials and Methods. Approximately 12 μg of intracisternal A particle protein (MOPC-104E) and $Mn^{2+}$ instead of $Mg^{2+}$ were used in each assay at 30 C. A particle DNA polymerase reaction was linear up to 40 min in these experiments. Specific activity for each isotope was: $^3$H-TTP, 12,900 counts per min per pmol; $^3$H-dCMP, 4,080 counts per min per pmol; $^3$H-dGMP, 2,560 counts per min per pmole. For each assay 5 μCi of isotope was used.

[b] Incorporation of $^3$H-TMP.

[c] Incorporation of $^3$H-dCMP.

[d] Incorporation of $^3$H-dGMP.

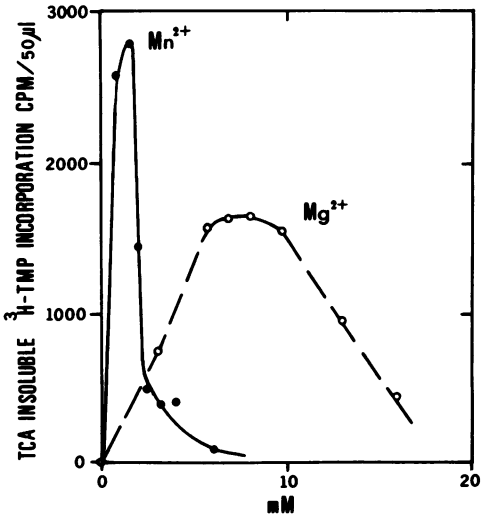


FIG. 5. *Optimal concentration of divalent cation required for activation of A particle RNA-dependent DNA polymerase. All assay conditions were described in Methods and Materials.*

ase of intracisternal A particles. Both the optimal concentrations for $Mg^{2+}$ and for $Mn^{2+}$ were found similar to those reported for murine and avian C-type viral reverse transcriptases (9, 13, 23). The $Mn^{2+}$ preference of intracisternal A particle endogenous RNA-dependent DNA polymerase suggests that it resembles the viral reverse transcriptase, since the stimulation by $Mn^{2+}$ is a characteristic of most viral reverse transcriptases (9, 10, 13, 23). It is noteworthy that $Mn^{2+}$ actually inhibits cellular DNA polymerase (10). In this respect the endogenous RNA-dependent DNA polymerase of purified intracisternal A particles differs significantly from the poly dT polymerase (32) which resembled the cellular enzyme associated with murine tissue culture cells described by Weissbach et al. (10, 31) by virtue of $Mg^{2+}$ preference.

**Analysis of the products of the endogenous RNA-dependent DNA polymerase reaction.** The nature of the DNA products differed depending on the duration of the endogenous DNA polymerase reaction as shown in the composite $Cs_2SO_4$ analyses (Fig. 6). The endogenous reaction of the purified intracisternal A particles used in these experiments was linear up to about 30 to 40 min. The $^3$H-DNA synthesized at the end of the first 5 min was clearly linked to the endogenous RNA; depending on the proportion of the newly synthesized DNA to endogenous RNA, the product sedimented at the RNA density of 1.65 g/cm$^3$ and at the

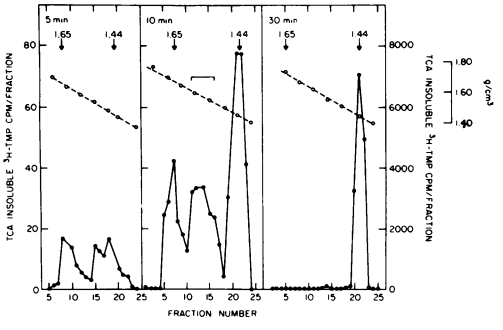


FIG. 6. *Analysis in $Cs_2SO_4$ gradients of the products of the endogenous reaction of intracisternal A particle DNA polymerase. About 2.0 mg of purified intracisternal A particles were assayed for endogenous activity at 37 C with 100 μCi of $^3$H-TTP (12.960 counts per min per pmol) and 8 × $10^{-5}$ M of all three other deoxyribonucleoside triphosphates in a final volume of 1.0 ml. Samples of 200 μliters each were withdrawn at 5 and 10 min, and the remaining 600 μliters were withdrawn at 30 min. The reaction was terminated by phenol-SDS extraction of the radioactive DNA product. Purification of the product and the $Cs_2SO_4$ analysis were described in Materials and Methods.*

DNA-RNA hybrid region from 1.50 to 1.55 g/cm$^3$ at a ratio of 42:58 with respect to the distributions of acid-insoluble $^3$H-DNA counts. At the end of 10 min of endogenous reaction, the $^3$H-DNA counts shifted more towards the hybrid region (1.55 g/cm$^3$) and the single-stranded DNA region of 1.44 g/cm$^3$ (17, 30), although some counts were still associated with the endogenous RNA (1.65 g/cm$^3$). The ratio of distribution of the $^3$H-DNA counts in the RNA region versus the hybrid and DNA regions was now about 25:75. At the end of 30 min of the endogenous reaction, the $^3$H-DNA product was found to be primarily free DNA. The product sedimented sharply at 1.44 g/cm$^3$ with only some overlapping at the 1.42 g/cm$^3$ area.

This product was found totally sensitive to DNAase digestion, and hybridized primarily with the high molecular weight RNA from intracisternal A particles. The pattern of appearance of the newly synthesized DNA products of the endogenous RNA-dependent DNA polymerase of intracisternal A particles also resembles those reported for avian and murine C-type viruses (11, 17, 24, 27, 30).

**Hybridization analysis with S-1 exonuclease.** S-1 exonuclease degrades the unhybridized region of single-stranded DNA to acid-soluble counts. Its action seems to be specific for the single-stranded DNA and the enzyme does not digest the RNA-DNA hybrid region (3). Table 3 summarizes the result in a hybridization analysis of the $^3$H-DNA product of the endogenous RNA-dependent DNA polymerase reaction of intracisternal A particles with the purified RNAs from the same source. Approximately 69 and 88% of the $^3$H-DNA synthesized in the presence of actinomycin D were found to hybridize back to the 60 to 70*S* RNA and 35*S* RNA of intracisternal A particles, respectively. The $^3$H-DNA product in its single stranded condition after alkali treatment at 100 C was totally degraded by S-1 exonuclease. Neither did it hybridize with heterologous RNA such as Qβ RNA. The DNA product of the endogenous RNA-dependent DNA polymerase of intracisternal A particles therefore showed a complementarity towards the high molecular weight RNA of the A particles. Detailed analysis of the nature of this DNA product synthesized by the A particle RNA-dependent DNA polymerase will be reported elsewhere.

## DISCUSSION

The success of demonstrating the association of an endogenous RNA-dependent DNA polymerase activity with intracisternal A particles

TABLE 3. *Hybridization analysis of $^3$H-DNA product of endogenous RNA-dependent DNA polymerase with RNAs of intracisternal A particles*

| Conditions | $^3$H-DNA bound to RNA | |
|---|---|---|
| | Counts per min per hybridization reaction[a] | Hybridized (%) |
| $^3$H-DNA input | 3,340 | 100 |
| $^3$H-DNA + S-1 exonuclease | 24 | 0 |
| ($^3$H-DNA-70*S* RNA) + S-1 exonuclease | 2,300 | 69 |
| ($^3$H-DNA-35*S* RNA) + S-1 exonuclease | 2,900 | 88 |
| ($^3$H-DNA + Q$\beta$ RNA) + S-1 exonuclease | 25 | 0 |

[a] All experimental conditions were described in Materials and Methods. Background count was 22 counts/min.

clearly depends on the nature of the particles at the end of the exhaustive isolation and purification procedures which free them from cytoplasmic cisternae. The technical pitfalls enumerated above all contributed to the observation of whether intracisternal A particles possess complete DNA polymerase activities with both endogenous RNA-directed reaction and a $dT_{12-18}$·poly rA-directed reaction or only a poly dT polymerase activity under the direction of $dT_{12-18}$·poly rA. To further complicate the technical problems, the replication of intracisternal A particles cannot be synchronized in either the MOPC-104E tumor tissues or the neuroblastoma N-18 tissue culture line. These A particles are continuously budding into the cisternae. The probability of obtaining open or budding forms of intracisternal A particles potentially deficient in high molecular weight RNA, and therefore endogenous RNA-dependent DNA polymerase activity, was great; such open forms have been described in a previous electron microscope study (15). Albeit, evidence thus far supports the fact that intracisternal A particles do possess an endogenous RNA-dependent DNA polymerase similar to other viral reverse transcriptases. It utilizes the A particle endogenous RNA and synthetic RNA-DNA hybrids as templates. It possesses characteristics typical of reverse transcriptase from oncornaviruses such as $Mn^{2+}$ preference, a template ratio of $dT_{12-18}$·poly rA/$dT_{12-18}$·poly dA greater than 9.9, and a moderate stimulation by $dG_{12-18}$·poly rC. The purified DNA products of the endogenous reaction synthesized in the presence of actinomycin D showed 70 to 90% base complementarity with the A particle 35 and 70*S* RNA. Currently, we are examining the homology of the DNA products of intracisternal A particle RNA-dependent DNA polymerase with high molecular weight RNAs of several C-type oncornaviruses. It is possible that the information derived will demonstrate more definitively the genetic relationship of intracisternal A particles with other RNA tumor viruses.

## ACKNOWLEDGMENTS

We thank Marian Smith and Harold Peart for their excellent technical assistance. We especially appreciate the helpful suggestions from David Baltimore in the preparation of this manuscript.

## LITERATURE CITED

1. Abrell, J. W., and R. C. Gallo. 1973. Purification, characterization and comparison of the DNA polymerases from two primate RNA tumor viruses. J. Virol. **12**:431-439.
2. Baltimore, D. 1970. Viral RNA-dependent DNA polymerase. Nature (London) **226**:1209-1211.
3. Benveniste, R. E., and E. M. Scolnick. 1973. RNA in mammalian sarcoma virus transformed non-producer cells homologous to murine leukemia virus RNA. Virology **51**:370-382.
4. Bolognesi, D. P., H. Gelderblom, H. Bauer, K. Mölling, and G. Huper. 1972. Polypeptides of avian RNA tumor viruses. V. Analysis of virus core. Virology **47**:567-578.
5. Calarco, P. G., and D. Szollosi. 1973. Intracisternal A particles in ova and preimplantation stages of the mouse. Nature N. Biol. **243**:91-93.
6. Dalton, A. J., and M. D. Felix. 1956. The electron microscopy of normal and malignant cells. N. Y. Acad. Sci. **63**:1117-1140.
7. Dalton, A. J., M. Potter, and R. M. Merwin. 1961. Some ultrastructural characteristics of a series of primary and transplanted plasma-cell tumors of the mouse. J. Nat. Cancer Inst. **26**:1221-1267.
8. DeHarven, E., and C. Friend. 1958. Electron microscopy study of a cell-free induced leukemia of the mouse. J. Biophys. Biochem. Cytol. **4**:151-156.
9. Faras, A. J., J. M. Taylor, J. P. McDonnell, W. E. Levinson, and J. M. Bishop. 1972. Purification and characterization of the deoxyribonucleic acid polymerase associated with Rous sarcoma virus. Biochemistry **11**:2334-2342.
10. Fry, M., and A. Weissbach. 1973. The utilization of synthetic ribonucleic acid template by a new deoxyribonucleic acid polymerase from cultured murine cells. J. Biol. Chem. **248**:2678-2683.
11. Fujinaga, K., J. T. Parsons, J. W. Beard, D. Beard, and M. Green. 1970. Mechanism of carcinogenesis by RNA tumor viruses. III. Formation of RNA·DNA complex and duplex DNA molecules by the DNA polymerases of avian myeloblastosis virus. Proc. Nat. Acad. Sci. U.S.A. **67**:1432-1439.
12. Garapin, A. C., J. P. McDonnell, W. Levinson, N. Quintrell, L. Fanshier, and J. M. Bishop. 1970. Deoxyribonucleic acid polymerase associated with Rous sarcoma virus and avian myeloblastosis virus: properties of the enzyme and its product. J. Virol. **6**:589-598.
13. Green, M., M. Rokutanda, K. Fujinaga, R. K. Ray, H. Rokutanda, and C. Gurgo. 1970. Mechanism of carcinogenesis by RNA tumor viruses. I. An RNA-dependent DNA polymerase in murine sarcoma viruses. Proc. Nat. Acad. Sci. U.S.A. **67**:385-393.

14. Kuff, E. L., K. K. Lueders, H. L. Ozer, and N. A. Wivel. 1972. Some structural and antigenic properties of intracisternal A particles occurring in mouse tumors. Proc. Nat. Acad. Sci. U.S.A. **69**:218-222.
15. Kuff, E. L., N. A. Wivel, and K. K. Lueders. 1968. The extraction of intracisternal A particles from a mouse plasma-cell tumor. Cancer Res. **28**:2137-2148.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. **193**:265-275.
17. Manly, K. F., D. F. Smoler, E. Bromfeld, and D. Baltimore. 1971. Forms of deoxyribonucleic acid produced by virions of the ribonucleic acid tumor viruses. J. Virol. **7**:106-111.
18. Nadel, E. W., W. Banfield, S. Burstein, and A. J. Tousimis. 1967. Virus particles associated with strain 2 guinea pig leukemia (L2C/N-B). J. Nat. Cancer Inst. **38**:979-981.
19. Novikoff, A. J., and L. Biempica. 1966. Cytochemical and electron microscopic examination of Morris 5123 and Reuber H-35 hepatomas after several years of transplantation p. 65-87. GANN Monogr. no. 1.
20. Sarin, P. S., and R. C. Gallo. 1973. RNA directed DNA polymerase, p. 1. *In* K. Burton (ed.), International review of science series in biochemistry, vol. 6, Nucleic acids. Butterworth Medical and Technical Publishing Co., Oxford.
21. Schlabach, A., B. Friedlander, A. Bolder, and A. Weissbach. 1971. DNA-dependent DNA polymerases from HeLa cell nuclei. II. Template and substrate utilization. Biochem. Biophys. Res. Commun. **44**:879-885.
22. Scolnick, E. M., W. P. Parks, G. J. Todaro, and S. A. Aaronson. 1972. Immunological characterization of primate C-type virus reverse transcriptase. Nature N. Biol. **235**:35-40.
23. Scolnick, E., E. Rands, S. A. Aaronson, and G. J. Todaro. 1970. RNA-dependent DNA polymerase activity in five RNA viruses: divalent cation requirements. Proc. Nat. Acad. Sci. U.S.A. **67**:1789-1796.
24. Spiegelman, S., A. Burny, M. R. Das, J. Keydar, J. Schlom, M. Travnicek, and K. Watson. 1970. Characterization of the products of RNA-directed DNA polymerases in oncogenic RNA viruses. Nature (London) **227**:563-567.
25. Stewart, S. E., G. Kasnic, C. Draycott, and T. Ben. 1972. Activation of viruses in human tumors by 5-iododeoxyuridine and dimethyl sulfoxide. Science **175**:198-199.
26. Sutton, W. D. 1971. A crude nuclease preparation suitable for use in DNA reassociation experiments. Biochem. Biophys. Acta **240**:522-531.
27. Taylor, J. M., A. J. Faras, H. E. Varmus, W. E. Levinson, and J. M. Bishop. 1972. Ribonucleic acid directed deoxyribonucleic acid synthesis by the purified deoxyribonucleic acid polymerase of Rous sarcoma virus. Characterization of the enzymatic product. Biochemistry **11**:2343-2351.
28. Temin, H. M., and S. Mizutani. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature (London) **226**:1211-1213.
29. Tumilowicz, J. J., and J. J. Cholon. 1971. Intracisternal type A particles and properties of a continuous cell line originating from a gerbil fibroma. Proc. Soc. Exp. Biol. Med. **136**:1107-1110.
30. Verma, I. M., N. L. Meuth, E. Bromfeld, K. F. Manly, and D. Baltimore. 1971. Covalently linked RNA-DNA molecule as initial product of RNA tumor virus DNA polymerase. Nature (London) **233**:131-134.
31. Weissbach, A., S. Schlabach, B. Friedlander, and A. Bolden. 1971. DNA polymerases from human cells. Nature N. Biol. **231**:167-170.
32. Wilson, S. H., and E. L. Kuff. 1972. A novel DNA polymerase activity found in association with intracisternal A particles. Proc. Nat. Acad. Sci. U.S.A. **69**:1531-1536.
33. Wivel, N. A., and G. H. Smith. 1971. Distribution of intracisternal A particles in a variety of normal and neoplastic mouse tissues. Int. J. Cancer **7**:167-175.
34. Yang, S. S., F. M. Herrera, R. G. Smith, M. S. Reitz, G. Lancini, R. C. Ting, and R. C. Gallo. 1972. Rifamycin antibiotics: inhibitors of Rauscher murine leukemia virus reverse transcriptase and of purified DNA polymerases from human normal and leukemic lymphoblasts. J. Nat. Cancer Inst. **49**:7-25.
35. Yang, S. S., and N. A. Wivel. 1973. Analysis of high-molecular-weight ribonucleic acid associated with intracisternal A particles. J. Virol. **11**:287-298.