

# Synthesis of extensive, possibly complete, DNA copies of poliovirus RNA in high yields and at high specific activities

(RNA-directed DNA nucleotidyltransferase/complementary DNA/avian myeloblastosis virus/formamide-polyacrylamide gel electrophoresis/RNA-DNA hybridization)

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**ABSTRACT** The synthesis of large, possibly complete, complementary DNA (cDNA) copies of poliovirus RNA by avian myeloblastosis virus DNA polymerase is described. The cDNA consists of two size classes, the larger of which is approximately 7500 nucleotides. In the presence of excess deoxynucleoside triphosphates, ribonucleoside triphosphates, or sodium pyrophosphate, only the larger material is obtained. Yields of the large cDNA are 50-75% of the input RNA.

The ability of reverse transcriptase to make highly-labeled complementary DNA (cDNA) copies of a variety of RNA templates has made available an extremely useful reagent for biological research. Hybridization experiments with cDNAs have been used to determine the number of genes coding for globin, immunoglobulin, and ovalbumin, to study the genetic defect in the thalassemias, to monitor messenger RNA synthesis and control, and to examine events in the life cycle of RNA tumor viruses (1).

Many investigators have reported attempts to synthesize complete, intact cDNAs from large, polycistronic messenger RNAs such as are found in the oncornavirus particle (1). The product DNA has always been considerably smaller than the template; and although in some cases there is faithful representation of the sequence information in the RNA, frequently a small portion of the template is represented in a greatly disproportionate amount.

The advantages of having complete, intact cDNAs are many. Sequence representation would be uniform and identical to that of the template. The powerful technologies being developed for DNA sequencing and for genetic analysis using restriction nucleases (2, 3) would be available for studying RNA viruses and messenger RNAs. cDNAs synthesized from mixed messenger RNA populations, e.g.,  $\alpha$  and  $\beta$  globin mRNAs, could be fractionated by means of specific restriction cuts. The purity of cDNA populations could be quickly and accurately assessed by examination of restriction nuclease fragment patterns on polyacrylamide gels. Complementary DNAs inserted into bacterial plasmids (4-6) could be used to synthesize useful quantities of DNA, RNA or protein to study systems where these cannot normally be obtained in the desired amounts.

We have been studying the mechanism of DNA synthesis by the reverse transcriptase (RNA-dependent DNA nucleotidyltransferase) of avian myeloblastosis virus (AMV) to determine what factors are responsible for the apparent failure of the enzyme to synthesize complete copies with large RNA templates. We report here that the polymerase is in our hands able to make extensive, possibly complete, complementary DNAs from poliovirus RNA in high yields and with specific activities suitable for use in hybridization and sequencing studies.

Abbreviations: PCC, phenol-cresol-chloroform mixture; AMV, avian myeloblastosis virus.

## MATERIALS AND METHODS

Unlabeled deoxyribonucleoside triphosphates and oligo(dT)<sub>10</sub> were purchased from P-L Biochemicals. [<sup>3</sup>H]dCTP and Na<sup>125</sup>I were obtained from New England Nuclear. Avian myeloblastosis virus was supplied by Dr. J. Beard, Life Sciences, Inc., St. Petersburg, Fla., through the Office of Resources and Logistics, Special Virus Cancer Program.

**Preparation of Poliovirus RNA.** Poliovirus (Mahoney Type I) was grown in HeLa S3 cells essentially as described by Summers (7) from stocks generously provided by Drs. E. Ehrenfeld and H. Oppermann. The virus was purified by standard procedures including treatment with sodium dodecyl sulfate and isopycnic centrifugation in CsCl gradients, basically as detailed by Roy and Bishop (8). The material from the gradients was pooled, diluted severalfold with 0.01 M Tris-HCl at pH 8.3, 0.15 M NaCl, 0.005 M EDTA (TNE buffer), and pelleted in the Beckman 60Ti rotor at 50,000 rpm for 2 hr at 4°. The virus (8.1 mg) was suspended in 25 ml of TNE and adjusted to 1.0% (wt/vol) sodium dodecyl sulfate. An equal volume of phenol-cresol-chloroform (PCC) prepared according to Kacian and Spiegelman (9) was added, and the mixture was shaken at room temperature for 30 min. The emulsion was separated by centrifugation in the Sorvall HB-4 rotor at 10,000 rpm for 10 min at 2°, and the aqueous phase was removed. It was brought to 0.4 M NaCl and extracted twice more with PCC. Two volumes of absolute ethanol were added, and the RNA was allowed to precipitate overnight at -20°. After centrifugation at 10,000 rpm in the HB-4 rotor at 0° for 30 min, the pellet was resuspended in 70% (vol/vol) ethanol and recentrifuged. The RNA was dissolved in 1.5 ml of distilled water and lyophilized. The yield was 2.8 mg. A<sub>260</sub>/A<sub>280</sub> ratio was 2.2.

**Polyacrylamide Gel Electrophoresis.** Polyacrylamide gel electrophoresis was performed in 3.5% gels prepared in 98% formamide according to Duesberg and Vogt (10). The gels were stained with "stains-all" as described by Dahlberg *et al.* (11) and scanned at 550 nm in a Gilford 2400S spectrophotometer.

**Alkaline Sucrose Density Gradient Centrifugation.** The approximate size of cDNAs was determined by centrifugation in 15-30% (wt/vol) sucrose gradients containing 0.9 M NaCl and 0.1 M NaOH in the Beckman SW56 rotor at 50,000 rpm for 12 hr at 1°. The ionic conditions are those described by Studier (12) who derived expressions relating the sedimentation coefficient and molecular weight of DNAs when so run.  $\phi$ X174am3 DNA (18.3S) was used as a marker. Gradients were fractionated dropwise from the bottom of the tube, and acid-precipitable radioactivity was collected on membrane filters.

**Preparation of  $\phi$ X174am3 DNA.**  $\phi$ X174am3 was grown as described (13) from stocks kindly provided by Dr. C. Hutchison

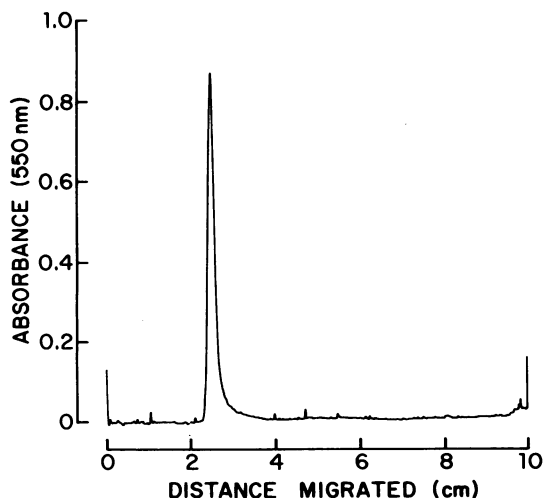


FIG. 1. Polyacrylamide gel in 98% formamide of polio RNA template. Two micrograms of polio RNA were run on a 3.5% gel as described in *Materials and Methods*. The gel was 10 cm long and was run at 50 V for 18 hr.

III. The phage DNA was prepared by an unpublished procedure (D. L. Kacian).

**Preparation of Avian Myeloblastosis Virus Reverse Transcriptase.** The isolation of the enzyme has been described previously (9, 14).

**Synthesis of cDNA.** Reverse transcriptase reaction mixtures contained the following: 50 mM Tris-HCl (pH 8.3), 8 mM MgCl<sub>2</sub>, 0.4 mM dithiothreitol, 50 mM KCl, 0.2 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM [<sup>3</sup>H]dCTP (5 Ci/mmol), 5 μg/ml of oligo(dT)<sub>10</sub>, 50 μg/ml of polio RNA, and 45 units/ml (9) of AMV reverse transcriptase.

Reactions were incubated at 37° for times given in the figures. For determination of acid-precipitable radioactivity, aliquots were diluted 200-fold into 1 mM EDTA at pH 7.0. An equal volume of trichloroacetic acid (TCA) reagent (9) was added and after 10 min at 0°, the material was collected on nitrocellulose membrane filters (S & S Type B-6). For alkaline sucrose gradient analysis, aliquots were diluted 75-fold in 1 mM EDTA and layered onto the gradients. Prior extraction of the reaction mixture with PCC-sodium lauroyl sarcosinate or extraction followed by alkaline hydrolysis of the RNA did not change the sedimentation properties of the cDNA.

**Preparation of <sup>125</sup>I-Labeled Polio RNA.** Polio RNA was labeled with <sup>125</sup>I according to methods described by Comerford (15) and Tereba and McCarthy (16). The specific activity of the RNA was 1.7 × 10<sup>7</sup> cpm/μg (gamma counter).

**Hybridization of cDNA to RNA.** Hybridizations were performed in 20 μl volumes in glass capillaries. The reaction mixture contained 20 mM sodium phosphate, pH 7.0, 300 mM NaCl, 2 mM EDTA, 0.1% (wt/vol) sodium dodecyl sulfate, 0.2 μg of either polio or total *Escherichia coli* RNA, and 1–2.3 ng of cDNA. The samples were heated at 100° for 2–3 min and incubated at 68° for 2 hr to attain C<sub>RNA-t</sub> (product of RNA concentration and time of incubation) = 0.2 mol of nucleotide-liter<sup>-1</sup>-sec. Reactions were terminated by freezing.

Hybridization was measured by a modification of the method of Kacian and Spiegelman (17). Two aliquots of 8 μl were taken from the hybridization reaction into nuclease reaction mixtures (200 μl final volume) containing 200 mM NaCl, 50 mM Tris-HCl at pH 8.3, 1 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 100 μg/ml of bovine serum albumin, and 0.12 μg/ml of denatured *E. coli* DNA. To one reaction mixture micrococcal nuclease was added to 4 units/ml; the other served as an incubated control without

nuclease. After incubation at 45° for 35 min, acid-precipitable radioactivity was collected on membrane filters.

**Ribonuclease Resistance of <sup>125</sup>I-Labeled Polio RNA Hybridized to Polio cDNA.** Hybridization was performed under the same conditions described above except that the amount of RNA used in various experiments ranged from 2.4 to 11.5 ng and the amount of cDNA was as indicated in Table 2. Reactions were incubated to attain C<sub>RNA-t</sub> ≥ 0.1 mol of nucleotide-liter<sup>-1</sup>-sec. Reactions were terminated as above and added to nuclease reaction mixtures (total volume 300 μl) containing 300 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl at pH 7.4, and either 53 μg/ml of RNase A or 26.5 μg/ml each RNase A and T1. Reactions containing only the one enzyme were incubated at 37° for 30 min; those with both enzymes were incubated at the same temperature for 1 hr. Acid-precipitable radioactivity was collected on membrane filters and measured in a gamma counter. Control reactions run in parallel were used to determine recovery, which was always at least 98%.

## RESULTS

There are several possible explanations for the failure of the AMV reverse transcriptase to synthesize complete, intact cDNA from large, polycistronic RNA templates. In many cases, the RNA offered the enzyme was probably degraded. Bader and Steck (18) and Erikson (19) pointed out that 60–70S RNA isolated from RNA tumor viruses contains substantial numbers of hidden breaks unless prepared from virus harvested at short intervals. Even if a portion of the template preparation is intact, breakdown products are frequently present in much higher molar amounts and may effectively compete with the undegraded RNA for the enzyme.

Degradative activities may be present. They may be expressed only under actual reaction conditions, which are frequently not the same as those used to check routinely for nuclease contamination. In addition, nucleases specific for structures formed only during the course of synthesis may be present.

The enzyme might be incapable of copying through highly-structured regions of the RNA. In the viron, the RNA may exist in association with membranes or proteins that afford a favorable configuration for synthesis. Termination of synthesis could also result from the presence of a sequence or structure code to which the enzyme responds. Internal primer binding sites may cause initiations to occur throughout the length of the molecule; if the enzyme were incapable of displacing these products, short cDNAs would result.

The binding of the enzyme to the template RNA may be weak, perhaps due to β subunit damage during isolation (1). The polymerase may then simply fall off the template at random intervals after synthesis has begun. This list is not exhaustive, and other explanations may be imagined.

In order to logically approach the problem of distinguishing among these alternatives, it was necessary to produce large amounts of intact, polycistronic RNA template that could be labeled at reasonable specific activities. We chose to use poliovirus RNA for these studies since it is large (approximately 7500 nucleotides) (20), has a poly(A) stretch at the 3' end (21, 22) for the binding of the oligo(dT)<sub>10</sub> primer, may have considerable secondary structure, is polycistronic, and can be easily prepared in intact form.

Fig. 1 shows a 3.5% polyacrylamide gel in 98% formamide of the unlabeled polio RNA used for the studies described here. The nucleic acid was prepared as described in *Materials and Methods* and applied to the gel without further fractionation.

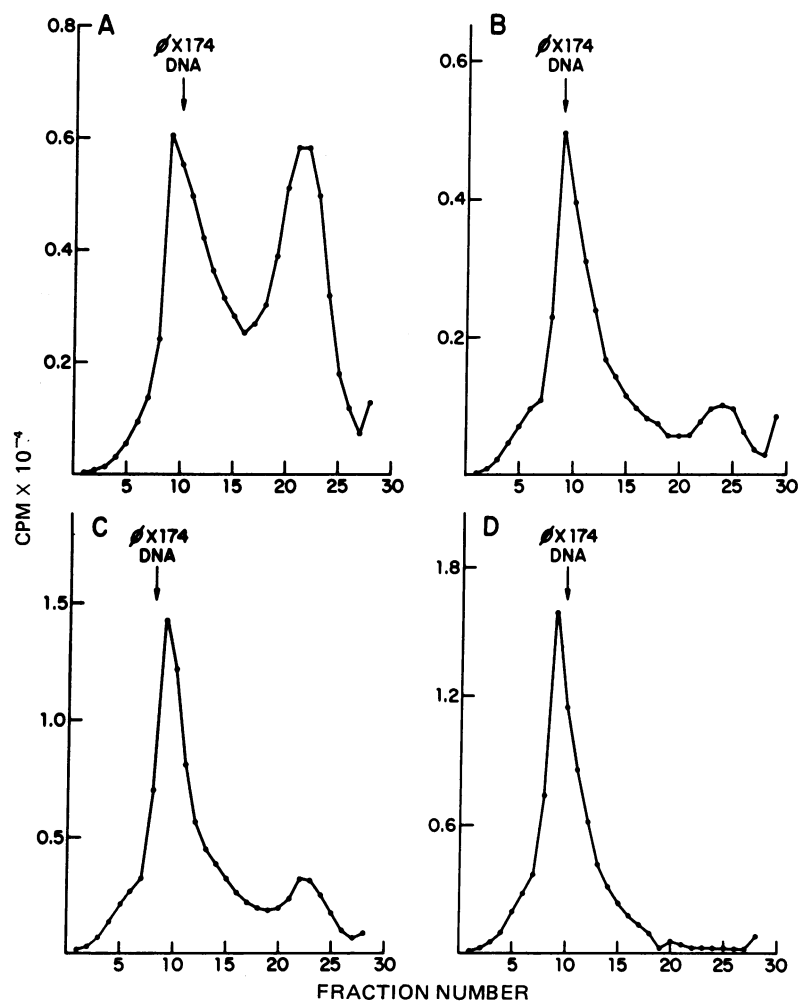


FIG. 2. Alkaline sucrose gradient centrifugation analysis of cDNAs made with polio RNA template. A, cDNA synthesized under standard reaction conditions as described in *Materials and Methods*; B, cDNA synthesized under standard reaction conditions + 1 mM each deoxyribonucleoside triphosphate; C, cDNA synthesized under standard reaction conditions + 1 mM each ribonucleoside triphosphate; D, cDNA synthesized under standard reaction conditions + 4 mM sodium pyrophosphate. All reactions were incubated for 2 hr. The position of  $\phi$ X174 DNA run on a parallel gradient is shown.

The RNA is seen to be substantially intact and contains only a small proportion of degraded or nonviral material.

When it was offered to the AMV reverse transcriptase under our standard reaction conditions, the cDNA shown in Fig. 2A was obtained. Two size classes of DNA were resolved on alkaline sucrose density gradients. The largest sediments together with  $\phi$ X174 DNA run in a parallel gradient. We chose the phage DNA as a marker for these studies since it sediments at 18.3 S under the given conditions. A complementary DNA of 7500 nucleotides, representative of the entire poliovirus genome, would sediment at 18.7 S; therefore, the largest class of cDNA seen in the gradient may be a complete or nearly complete copy of the template RNA.

Sucrose gradient centrifugation under these conditions is inadequate to determine whether or not synthesis has in fact proceeded to the 5' end of the RNA. cDNAs of one-half the polio genome (about 3750 nucleotides) would sediment at 14.2 S, whereas those of 9000 nucleotides would run at 20.1 S. We have, therefore, only included a single marker of the appropriate size in these gradients, recognizing that the value obtained is, in any event, only approximate. Even more careful sizing of the cDNA would not determine whether transcription is complete since the size of the RNA template has been mea-

sured from 2 to  $2.7 \times 10^6$  daltons. Studies are currently in progress to show whether or not the 5' terminus of the polio RNA is represented in the larger cDNA population.

In addition to the 18S cDNA, a considerable amount of small cDNA is found. A more detailed analysis of the reaction using [ $^3$ H]polio RNA template and [ $^{32}$ P]dCTP shows that this smaller material is synthesized later in the reaction, essentially after synthesis of the large material has ceased. In addition, the template RNA is degraded as synthesis proceeds, and it may be that the smaller material arises as a consequence of transcription from the fragmented template. These studies are too extensive to be presented here, and they will be published elsewhere.

After observing that a significant amount of large cDNA synthesis would occur when intact RNA was provided to the enzyme, and that template degradation occurs contemporaneously with small cDNA synthesis, we decided to explore the effects of adding nuclease inhibitors to the polymerase reaction. We wished to select inhibitors that would not affect the synthetic reaction, if possible, and we therefore rejected classic additives such as polyvinyl sulfate and other anionic polymers, which are also potent inhibitors of reverse transcriptase activity. It is well-known that, frequently, phosphate ion and nucleotides

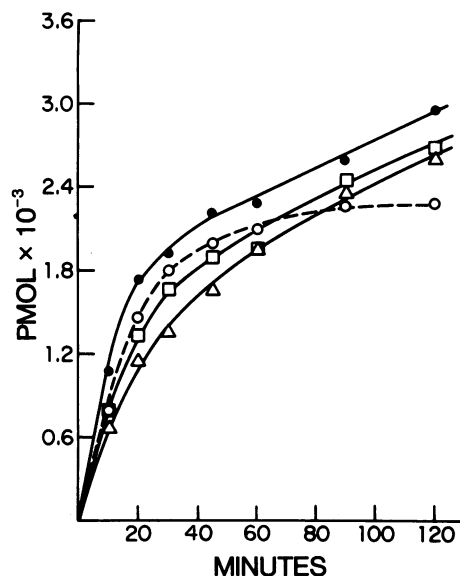


FIG. 3. Kinetics of cDNA synthesis with polio RNA template. Reactions were run under the same conditions described in the legend to Fig. 2. The ordinate gives pmol of dCMP incorporated per 0.1 ml reaction. (—  $\Delta$  —), standard reaction; (—  $\bullet$  —), standard reaction + 1 mM each deoxyribonucleoside triphosphate; (—  $\square$  —), standard reaction + 1 mM each ribonucleoside triphosphate; (—  $\circ$  —), standard reaction + 4 mM sodium pyrophosphate.

are potent inhibitors of nucleases, and we knew from previous attempts to elicit RNA-directed RNA synthesis from the enzyme that ribonucleotides were well-tolerated. We therefore examined the effects of adding deoxyribonucleoside mono- and triphosphates, ribonucleoside mono- and triphosphates, sodium pyrophosphate, and sodium phosphate to the enzyme reaction. The size of the cDNA obtained with several of these additives is shown in Figs. 2B–D. Addition of either deoxy- or ribonucleoside triphosphates or sodium pyrophosphate resulted in significant repression of small cDNA synthesis. In the presence of the latter agent, essentially all of the product made was of the larger size. Addition of nucleoside monophosphates or sodium phosphate at equivalent levels was without effect (results not shown); therefore, it appears that effective inhibitors of small cDNA synthesis are drawn from among those compounds containing phosphodiester bonds. A number of workers have observed an increase in overall cDNA size when higher deoxyribonucleoside triphosphate concentrations were used (23–26); however, only a small amount, if any, intact product was synthesized from large, polycistronic RNAs.

Fig. 3 shows the kinetics of synthesis in a control reaction performed under our standard conditions and in the presence of deoxy- and ribonucleoside triphosphates and sodium pyrophosphate. Addition of the inhibitors results in an increase in the rate of the reaction as well as a diminution of smaller cDNA synthesis. The polymerase reaction continues in most cases for at least 2 hr, but when pyrophosphate is added it ceases at earlier times. This observation is consistent with data indicating that synthesis of small cDNA occurs late in the reaction (Kacian and Myers, in preparation) since little such material is made in the presence of that additive.

In order to verify that the material being made in these reactions is indeed DNA complementary to poliovirus RNA, we tested its ability to anneal to the template. Table 1 shows that over 95% of the large cDNAs made in the presence of all three additives hybridize back to the RNA template. In all of these reactions, run in the absence of actinomycin D, synthesis ap-

Table 1. Hybridizability of polio cDNAs

Polio cDNA made in presence of	Percentage nuclease resistant*
High deoxyribonucleoside triphosphates	
+ Polio RNA	97 $\pm$ 3
+ <i>E. coli</i> RNA	3 $\pm$ 1
Ribonucleoside triphosphates	
+ Polio RNA	97 $\pm$ 5
+ <i>E. coli</i> RNA	6 $\pm$ 1
Sodium pyrophosphate	
+ Polio RNA	96 $\pm$ 2
+ <i>E. coli</i> RNA	4 $\pm$ 0

cDNAs were prepared as described in Fig. 2. The large peaks were pooled from the gradients, neutralized, passed through Sephadex G-50 columns and lyophilized as described in *Materials and Methods*.

\* Values are averages of two, or in some cases, three, determinations followed by the range of values obtained.

pears to be limited to single-stranded cDNA. This behavior has also been observed by Efstratiadis *et al.* (24) when globin cDNA was synthesized with high substrate concentrations. Apparently, under conditions where the enzyme makes extensive or complete copies, copying from the DNA strand does not occur.

The ability of the cDNA to protect its template from ribonuclease digestion is shown in Table 2. Polio RNA was labeled with  $^{125}\text{I}$  and annealed with various amounts of large cDNA. The hybrids were treated with ribonuclease in high salt, and the acid-precipitable radioactivity remaining was determined. The results indicate that the cDNA is able to protect 90% or more of the labeled template, indicating that it contains all, or

Table 2. RNase resistance of polio  $^{125}\text{I}$ -RNA-cDNA hybrids

cDNA* made in presence of	ng cDNA / ng RNA	Fraction RNase resistant
High deoxyribonucleoside triphosphates	0	0.10
	0.18	0.28
	0.35	0.42
	0.53	0.66
	1.83	0.95
Ribonucleoside triphosphates	0	0.10
	0.21	0.33
	0.41	0.57
	0.62	0.72
	1.83	0.94
Sodium pyrophosphate	0	0.02
	0.36	0.40
	0.72	0.71
	1.08	0.83
	1.44	0.90
	2.16	0.90

\* cDNAs were prepared as described in Table 1.

Experiments using cDNAs made in the presence of high deoxyribonucleoside triphosphates and ribonucleoside triphosphates were done with RNase A. In order to further reduce background levels, the experiments using cDNA made in the presence of sodium pyrophosphate were done with both RNase A and RNase T1.

nearly all, the sequences present in the RNA in approximately equivalent amounts.

### DISCUSSION

The results presented above show that the reverse transcriptase from AMV is able to synthesize a complete or nearly complete DNA copy from poliovirus RNA. Similar results have been obtained with intact Rous sarcoma virus RNA (Kacian and Myers, in preparation).

The concentrations of the inhibitors used are critical. Excessive concentrations depress the production of large cDNA and the total yield of the reaction. The maximum rate of synthesis and the maximum product size are obtained under the same conditions; therefore, it is possible to optimize both simply by measuring acid-precipitable radioactivity. As the concentration of the particular additive being used is increased, the rate and extent of cDNA synthesis will rise and then decline. If the template RNA is intact, the concentration giving maximum synthesis will also produce the largest cDNA. Raising the  $Mg^{++}$  from 8 mM to 20 mM considerably reduced the size of the cDNA. Nuclease levels should be assayed with the large templates themselves and with DNAs of comparable size for maximum sensitivity, and the sizes of the nucleic acids after incubation should be checked under denaturing conditions.

Since sodium pyrophosphate and ribonucleoside triphosphates are as effective as or more effective than deoxyribonucleoside triphosphates at inhibiting smaller cDNA synthesis, the products may be prepared without sacrificing high specific activity or using prohibitively large amounts of isotope. Yields are very reasonable. As seen in Fig. 3, synthesis equal to 50–75% of the input template is obtained (assuming equal incorporation of the four bases); thus, the synthesis of even milligram amounts of cDNA is feasible.

The apparent inability of the enzyme to synthesize complete double-stranded DNA has advantages and disadvantages. For hybridization purposes, single-stranded cDNA is usually desired, and this may be obtained without the lower yields obtained when actinomycin D is used to repress synthesis of the second strand. Double-stranded product would be preferred for work with restriction nucleases; however, Horiuchi and Zinder (27) have recently shown that *Hemophilus aegyptius* endonuclease *Hae*III will specifically cut single-stranded DNA with the same specificity when higher enzyme levels and longer incubation times are used than with the double-stranded substrate. Similar results have been obtained with a number of other restriction nucleases (28). For plasmid work, double-stranded DNA is essential. Whether it will be possible to coax the AMV enzyme into making it or whether more elaborate techniques will be required remains a subject for further study.

We are especially grateful to Dr. John Holland for a generous gift of  $^{32}P$ -labeled poliovirus, which enabled us to test the validity of our approach, and to Drs. E. Ehrenfeld and H. Oppermann for frequent and very helpful advice on growing poliovirus. We would also like to thank Dr. S. Spiegelman for his liberal support. This work was supported by National Institutes of Health Contract N01-CP-3-3258 and Grant CA-02332-21.

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