Model RNA-Directed DNA Synthesis by Avian Myeloblastosis Virus DNA Polymerase and Its Associated RNase H†

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ABSTRACT: A model RNA template·primer system is described for the study of RNA-directed double-stranded DNA synthesis by purified avian myeloblastosis virus DNA polymerase and its associated RNase H. In the presence of complementary RNA primer, oligo(rI), and the deoxyribonucleoside triphosphates dGTP, dTTP, and dATP, 3′-$(rC)_{30-40}$-poly(rA) directs the sequential synthesis of poly(dT) and poly(dA) from a specific site at the 3′ end of the RNA template. With this model RNA template·primer, optimal conditions for double-stranded DNA synthesis are described. Analysis of the kinetics of DNA synthesis shows that initially there is rapid synthesis of poly(dT). After a brief time lag, poly(dA) synthesis and the DNA polymerase–associated RNase H activity are initiated. While poly(rA) is directing the synthesis of poly(dT), the requirements for DNA synthesis indicate that the newly synthesized poly(dT) is acting as template for poly(dA) synthesis. Furthermore, selective inhibitor studies using NaF show that activation of RNase H is not just a time-related event, but is required for synthesis of the anticomplementary strand of DNA. To determine the specific role of RNase H in this synthetic sequence, the primer for poly(dA) synthesis was investigated. By use of formamide–polyacrylamide slab gel electrophoresis, it is shown that poly(dT) is not acting as both template and primer for poly(dA) synthesis since no poly(dT)–poly(dA) covalent linkages are observed in radioactive poly(dA) product. Identification of 2′,3′-[^{32}P]AMP on paper chromatograms of alkali-treated poly(dA) product synthesized with [α-^{32}P]dATP as substrate demonstrates the presence of rAMP–dAMP phosphodiester linkages in the poly(dA) product. Therefore, a new functional role of RNase H is demonstrated in the RNA-directed synthesis of double-stranded DNA. Not only is RNase H responsible for the degradation of poly(rA) following formation of a poly(rA)·poly(dT) hybrid but also the poly(rA) fragments generated are serving as primers for initiation of synthesis of the second strand of the double-stranded DNA.

Avian myeloblastosis virus DNA polymerase catalyzes the synthesis of cDNA[1] from a variety of natural (Spiegelman et al., 1971; Verma et al., 1972; Kacian et al., 1972; Ross et al., 1972; Leis & Hurwitz, 1972; Green & Gerard, 1974) and synthetic RNA template·primers (Spiegelman et al., 1970; Scolnick et al., 1970; Baltimore & Smoler, 1971; Goodman & Spiegelman, 1971; Leis & Hurwitz, 1972; Wells et al., 1972; Green & Gerard, 1974). One synthetic ribopolymer, poly(rA), has been particularly effective for elucidating the process of initiation and elongation of the RNA-directed synthesis of cDNA (Baltimore & Smoler, 1971; Goodman & Spiegelman, 1971; Smoler et al., 1971; Leis & Hurwitz, 1972). While synthesis of dsDNA has also been described, this information comes primarily from the more complex endogenous and reconstructed oncornavirus RNA-directed systems (Fujinaga et al., 1970; Garapin et al., 1970; Fanshier et al., 1971; Manly et al., 1971; Taylor et al., 1973; Leis & Hurwitz, 1972; Rothenberg et al., 1977), and the mechanism of its formation remains obscure. The role of the DNA polymerase–associated RNase H activity (Keller & Crouch, 1972; Baltimore & Smoler, 1972; Watson et al., 1973) in dsDNA synthesis is also unclear (Brewer & Wells, 1974; Collett & Faras, 1976; Kacian & Myers, 1976; Collett et al., 1978). However, Myers & Spiegelman (1978) have recently shown that inhibition of degradation of a DNA·RNA hybrid by AMV DNA polymerase–associated RNase H is coupled with the suppression of synthesis of anticomplementary DNA. From these results, they suggest that the inhibitor ($Na_4P_2O_7$) blocks the RNase H activity and further prevents the formation of RNA fragments necessary to prime the synthesis of anticomplementary DNA.

In developing a model in vitro system for further study of the events of RNA-directed DNA synthesis by using purified AMV DNA polymerase, we have asked whether a modified form of the synthetic ribopolymer, poly(rA), could act as an RNA template for synthesis of dsDNA and whether RNase H has a role in this synthetic process. In this report, we demonstrate that in the presence of a 3′-modified poly(rA) template·primer model, purified AMV DNA polymerase directs the sequential synthesis of two complementary strands of DNA. By use of a specific inhibitor of RNase H activity (NaF), it is shown that RNase H is required for initiation of synthesis of the anticomplementary strand of DNA. Finally, to establish that RNA template fragments generated by RNase H activity were acting as primers for initiation of anticomplementary DNA synthesis as previously suggested (Baltimore & Smoler, 1971; Keller & Crouch, 1972; Leis et al., 1973; Myers & Spiegelman, 1978), we demonstrate the presence of a covalent linkage between the RNA and anticomplementary DNA. These studies represent the first direct evidence that anticomplementary DNA strand synthesis by a retrovirus DNA polymerase is initiated on a RNA primer derived from the original template and indicate that the DNA polymerase–associated RNase H activity is responsible for

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[1] Abbreviations used: AMV, avian myeloblastosis virus; cDNA, complementary DNA; dsDNA, double-stranded DNA; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; dNTP, deoxyribonucleoside 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediamintetraacetic acid.

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generating the primers for this synthesis. Moreover, our results suggest the usefulness of such model RNA template·primer systems for elucidating the combined roles of these enzymic activities in the initiation and synthesis of RNA-directed double-stranded DNA.

Experimental Procedures

Reagents. All chemicals were reagent or enzyme grade. DEAE-cellulose paper disks (DE-81, 2.4-cm diameter) were obtained from Whatman, Inc., Clifton, NJ. *Micrococcus luteus* "primer-dependent" polynucleotide phosphorylase and poly(dT) (6.5 S) were obtained from P-L Biochemicals, Milwaukee, WI. ^{3}H- and ^{32}P-labeled dNTPs were products of ICN Corp., Irvine, CA. [^{3}H]CDP was obtained from New England Nuclear Corp., Boston, MA. Poly(rA) (7.7 S) was purchased from Miles Laboratories, Elkhart, IN. Oligo(dT)–cellulose, $(rI)_{10-20}$, $(dT)_{12-18}$, and $(rU)_{10-20}$ were from Collaborative Research, Waltham, MA. DNase I (electrophoretically purified) from bovine pancreas and crude α-amylase powder from *Aspergillus oryzae* were obtained from Sigma Chemical Co., St. Louis, MO.

Virus and Enzymes. AMV (BAI strain A) was extensively purified as previously described (Carnegie et al., 1969; Kacian et al., 1971) from frozen chick plasma supplied through the Office of Program Resources and Logistics, Virus Cancer Program, Viral Oncology Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, MD.

AMV DNA polymerase was solubilized from purified AMV essentially as previously described (Kacian et al., 1971) in a medium containing a final concentration of 6.7% Nonidet P-40, 0.8 M KCl, and 5 mg/mL viral protein. The mixture was kept at 0 °C for 45 min and then centrifuged at 20000*g* for 15 min at 1 °C. The supernatant was diluted 10-fold with 0.01 M K_2HPO_4 (pH 7.2), 3 mM dithiothreitol, and 5% glycerol and was applied to a DEAE-cellulose column (2 × 15 cm). The enzyme was further purified to apparent homogeneity as determined by $NaDodSO_4$–PAGE by using CM-Sephadex chromatography and glycerol gradient centrifugation (Kacian et al., 1971).

Nuclease S_1 was purified from crude α-amylase powder from *A. oryzae* through the DEAE-cellulose chromatography step by the method of Vogt (1973).

RNA polymerase from *Escherichia coli* MRE 600 was purified through the high- and low-salt velocity gradient centrifugation steps as described by Burgess (1969).

Standard Assay for AMV DNA Polymerase and Ribonuclease H. Standard reaction mixtures (0.1 mL) contained the following: 50 mM Tris-HCl (pH 8.2 at 20 °C); 40 mM KCl; 8 mM $MgCl_2$; 5 mM dithiothreitol; 0.2 mM dATP and dGTP; and 0.05 mM dTTP. [^{3}H]dATP (specific activity 70–100 cpm/pmol) was included in assays to monitor poly(dA) synthesis and [^{3}H]dTTP (specific activity 70–100 cpm/pmol) was included in assays to monitor poly(dT) synthesis. Addition of previously annealed $3'$-$(rC)_{30-40}$-$(rA)_{\overline{300}}$·$(rI)_{10-20}$ [400 pmol of AMP residues–20 pmol of IMP residues, representing a molar ratio of $3'$-$(rC)_{30-40}$-$(rA)_{\overline{300}}$·$(rI)_{10-20}$ of 1:1] and one unit of purified AMV DNA polymerase completed the standard reaction mixture. Annealing of template and primer was carried out by heating the mixtures (containing 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1 M NaCl) at 90 °C for 10 min followed by a 30-min incubation at 45 °C. One unit of DNA polymerase activity is defined as the amount of enzyme required to catalyze the incorporation of 1 pmol of [^{3}H]dTMP/min in the above reaction conditions. After incubation at 37 °C for the designated time, the reaction mixtures were placed in ice and aliquots were spotted on DE-81 ion-exchange cellulose filter paper disks and treated for counting as described previously (Blatti et al., 1970).

To monitor RNase H activity during DNA synthesis, we used $3'$-$(rC)_{30-40}$-poly(rA)·$(rI)_{10-20}$ as the template·primer. Radiolabeled poly(rA) degradation was measured by the loss of radiolabeled template binding to DE-81 ion-exchange cellulose filter paper disks as described above. Preparation and modification of radiolabeled poly(rA) are described elsewhere in Experimental Procedures.

Polynucleotide Phosphorylase Assay. Reaction mixtures (0.1 mL) contained the following: 100 mM Tris-HCl (pH 9.0 at 20 °C); 10 mM $MgCl_2$; 10 μg of bovine serum albumin; 20 mM [^{3}H]CDP (specific activity 10^3 cpm/nmol); 0.4 mM EDTA; 40 μg of $(rA)_{\overline{300}}$ primer; and 0.2 unit of *M. luteus* "primer-dependent" polynucleotide phosphorylase. One unit of polynucleotide phosphorylase activity catalyzed the polymerization of 1 μmol of ADP in 15 min at 37 °C in the presence of 3.3 A_{257} units of tetra(A) triphosphate primer/mL of reaction mixture, as measured by phosphate release. After incubation at 37 °C, reaction mixtures were terminated in ice and aliquots were spotted on DE-81 ion-exchange cellulose filter paper disks and treated for counting as previously described (Blatti et al., 1970).

Preparation of Radiolabeled Poly(rA). Radiolabeled poly(rA) was synthesized in vitro by utilizing *E. coli* RNA polymerase and poly(dT) as template. A reaction mixture (0.5 mL) was prepared and contained the following: 50 mM Tris-HCl (pH 7.9 at 25 °C); 0.8 mM $MnCl_2$; 0.1 mM EDTA; 5 mM dithiothreitol; 0.15 M NaCl; 50 μg of bovine serum albumin; 0.4 mM K_2HPO_4 (pH 8.0); 5% (v/v) glycerol; 0.15 mM [α-^{32}P]ATP or [^{3}H]ATP (5 × 10^3 cpm/pmol); 2 μg of poly(dT); and 5 μL of *E. coli* RNA polymerase (1 mg/mL of protein). After 90 min at 37 °C, the reaction was terminated with $NaDodSO_4$ [1% (w/v) final concentration], phenol-extracted, and precipitated overnight with 2 vol of ethanol at −20 °C. Poly(dT) was removed by DNase I digestion (0.1 mg/mL) of the product at 37 °C for 90 min followed by $NaDodSO_4$–phenol extraction and G-50 Sephadex chromatography in the absence of salt. The gel-excluded, radiolabeled poly(rA) was further concentrated by precipitation with 2 vol of ethanol at −20 °C and was demonstrated to be essentially free of oligo(dT) primer fragments by testing for poly(dT) synthesis in the absence of added $(dT)_{12-18}$ or $(rU)_{10-20}$ primer in a standard reverse transcriptase assay. The average length of the radioactive poly(rA) was calculated to be 100–150 AMP residues by using formamide–PAGE (Wang & Duesberg, 1974).

Preparation of 3′-Modified Poly(rA). Aliquots (0.75 mg) of commercial poly(rA) were centrifuged in 10–30% (v/v) glycerol gradients containing 10 mM Tris-HCl (pH 7.4), 3 mM EDTA, and 0.1 M NaCl for 23 h at 40000 rpm and 3 °C in a Spinco SW 41 rotor. After the gradient fractions (0.4 mL) were collected by needle puncture, the absorbancy at 260 nm of each fraction was determined. The 260-nm peak absorbing fraction from each gradient was combined into one pool, and an estimated molecular weight of the poly(rA) was determined to be 110000–120000 by using the formamide–PAGE method described by Wang & Duesberg (1974). From this estimated value, the average length of poly(rA) molecules was calculated to be 300–320 AMP residues. This $(rA)_{\overline{300}}$ fraction was then extended at its 3′-hydroxy terminus by the enzymatic addition of CMP residues by using *M. luteus* polynucleotide phosphorylase and unlabeled CDP as substrate under the conditions described for the polynucleotide phos-

phorylase assay. Upon addition of 40 μg of $(rA)_{\overline{300}}$ and 0.2 unit of polynucleotide phosphorylase, the reaction mixture (0.1 mL) was incubated for 60 min at 37 °C. For termination, the reaction mixture was made 0.5 M in NaCl, 5 mM in EDTA, and 0.2% (w/v) in $NaDodSO_4$. This mixture (0.4 mL) was applied to a 1-mL column of oligo(dT)–cellulose preequilibrated in 0.5 M NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), and 0.2% $NaDodSO_4$. The reaction tube was washed once with 0.5 mL of preequilibration buffer and added to the column. After the column was washed with 10 mL of preequilibration buffer, the 3′-modified $(rA)_{\overline{300}}$ was eluted in 1-mL fractions with 10 mL of buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.2% $NaDodSO_4$. Each fraction was monitored for absorbance at 260 nm. Greater than 95% of the input RNA was recovered in two fractions. After being pooled, the 3′-modified $(rA)_{\overline{300}}$ was concentrated by alcohol precipitation overnight at −20 °C.

Preparation of Oligo(rA) Fragments. Oligo(rA) fragments were products of nuclease S_1 digestion of poly(rA). Poly(rA) (3 mg) was treated at 42 °C for 1.5 h with 300 units of nuclease S_1 (1 unit of enzyme was sufficient to acid-solubilize 90% of 10 μg of heat-denatured ^{3}H-labeled *E. coli* DNA in 45 min at 40 °C) in the presence of 25 mM $NaC_2H_3O_2$, pH 4.5, 5 mM $ZnSO_4$, and 0.3 M NaCl. Following digestion, the pH of the mixture was adjusted to 7.5 and the mixture was chromatographed on DEAE-cellulose in the presence of 7 M urea as described by Bishop et al. (1968). The fractions containing fragments of 12–20 AMP residues in length were pooled, rechromatographed, and concentrated (Bishop et al., 1968). Oligo(rA) was then annealed with poly(dT) (20 pmol of AMP residues–400 pmol of dTMP residues) by using the same procedure described above for preparation of 3′-modified poly(rA)·oligo(rI) template·primer.

Formamide–PAGE and Fluorography of [^{3}H]DNA Products. Polyacrylamide slab gels (5% w/v) (10 × 14.2 × 0.12 cm) in 99% (v/v) formamide were prepared as described by Maniatis et al. (1975). Ethanol-precipitated [^{3}H]poly(dT) and [^{3}H]poly(dA) products were resuspended in 40 μL of formamide, heated at 80 °C for 2 min, and quick-cooled in an ice–water bath. Electrophoresis was performed at room temperature for 9 h at a constant voltage of 75 V.

Poly(rA) (5 μg) and Hae III restriction fragments of φX-174 DNA (2 μg) were used as nucleic acid markers and were detected after ethidium bromide staining (2.5 μg/mL) for 60 min. For fluorography of the [^{3}H]DNA products, the slab gel was first soaked in a 1% (w/v) lanthanum acetate–1% (v/v) glacial acetic acid solution for 1 h to fix the DNA and then the gel was washed sequentially with dimethyl sulfoxide and 20% (w/v) 2,5-diphenyloxazole (PPO) in dimethyl sulfoxide according to the method of Bonner & Laskey (1974). The gel was placed on Whatman 3MM paper, covered with Saran wrap, and dried. Kodak XR-1 film was exposed to the dried gel for 24–72 h at −70 °C in an X-ray exposure holder containing two X-ray intensifying screens.

Detection of rAMP–dAMP Covalent Linkages in Poly(dA) Product. A reaction mixture (0.3 mL) was prepared containing 50 mM Tris-HCl (pH 8.2), 60 mM KCl, 8 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 mM dGTP, 0.05 mM dTTP, $3'\text{-}(rC)_{30\text{-}40}\text{-}(rA)_{\overline{300}}\text{-}(rI)_{10\text{-}20}$ template·primer (1.2 nmol of rAMP residues and 60 pmol of rIMP residues), and 3 units of purified AMV DNA polymerase. After incubation for 15 min at 37 °C, [α-^{32}P]dATP was added to a final concentration of 0.05 mM (specific activity, 6 × 10^4 cpm/pmol). After an additional 10 min at 37 °C, the reaction was terminated by the addition of $NaDodSO_4$ to a final concentration of 0.2%, followed by phenol extraction and G-50 Sephadex chromatography for removal of excess radiolabeled substrate. After concentration of the DNA product by precipitation with 2 vol of ethanol at −20 °C, a 0.1-mL sample (3 × 10^5 cpm) was alkali-treated (0.3 M NaOH) for 18 h at 37 °C and neutralized with HCl. Ascending paper chromatography was performed on Whatman 3MM paper by using the solvent system isobutyric acid–concentrated NH_3–H_2O (57:4:39, v/v). After the paper was dried, sample strips were cut into 0.5-cm segments and counted in a liquid scintillation spectrometer. For synthesis of [^{32}P]poly(dA) using poly(dT)·oligo(rA) template·primer, identical conditions and treatment as described above were followed except that the [α-^{32}P]dATP was added to the initial reaction mixture and synthesis proceeded for 30 min at 37 °C.

Results

Modified Poly(rA) as Model RNA Template for dsDNA Synthesis. Our objective in this model study was to investigate the role of viral DNA polymerase and its associated RNase H activity in specific events of dsDNA synthesis. Because the formation of synthetic poly(rA)-containing RNA·RNA duplexes like poly(rA)·oligo(rU) is a random process which, very likely, gives rise to multiple sites for initiation of DNA synthesis on any one poly(rA) template molecule, it was advantageous to modify the poly(rA) template in such a way that initiation of cDNA synthesis was a controlled event with one primer molecule at a specified position on each RNA template. Then, with a uniformly sized poly(rA) template, elongation of cDNA and initiation of dsDNA synthesis should proceed in a more synchronous manner. To achieve this, sized poly(rA) was modified at its 3′ terminus by the addition of CMP residues in the presence of *M. luteus* "primer-dependent" polynucleotide phosphorylase and CDP as substrate. To determine the amount of enzyme necessary for extending the poly(rA) strands with 30–40 CMP residues each, we prepared reaction mixtures containing increasing amounts of polynucleotide phosphorylase in the presence and absence of poly(rA) primer and incubated them for 40 min at 37 °C. Throughout the range of enzyme concentration tested, there was an increasing response to added enzyme and the primer-independent incorporation of [^{3}H]CMP into polymer was less than 5% of the primer-dependent activity. Also, incorporation of [^{3}H]CMP was linear for more than 60 min at all enzyme levels tested. With poly(rA) primer molecules estimated to have an average length of 300–320 residues, and assuming all poly(rA) molecules were acting as primers, an estimate of the number of 3′ terminally added CMP residues per poly(rA) strand per minute was determined. In the presence of 0.2 unit of polynucleotide phosphorylase, ~0.6 residue of CMP was added per minute to each molecule of poly(rA). Thus, in 60 min, it was possible to extend each poly(rA) molecule with ~30–40 CMP residues. By use of these reaction conditions, larger quantities of $3'\text{-}(rC)_{30\text{-}40}\text{-}(rA)_{\overline{300}}$ template were prepared. Poly(rC) resulting from primer-independent activity of the *M. luteus* polynucleotide phosphorylase was separated from the 3′-modified poly(rA) by oligo(dT)–cellulose affinity chromatography.

Annealing of $(rI)_{10\text{-}20}$ to the 3′-terminal CMP residues of $3'\text{-}(rC)_{30\text{-}40}\text{-}(rA)_{\overline{300}}$ resulted in a functional template·primer for poly(rA)-directed poly(dT) synthesis by purified AMV DNA polymerase (Figure 1). Both the $3'\text{-}(rC)_{30\text{-}40}$ portion of the $3'\text{-}(rC)_{30\text{-}40}\text{-}(rA)_{\overline{300}}$ template and the oligo(rI) primer were essential for optimal poly(dT) synthesis, in that un-

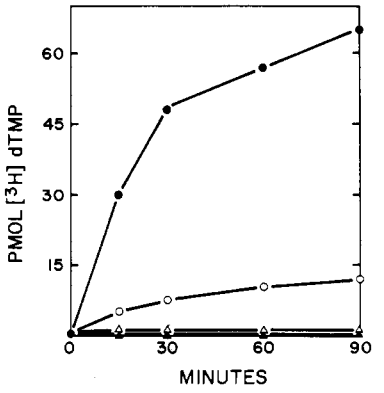

FIGURE 1: Kinetics and template·primer requirements of 3′-$(rC)_{30-40}$-poly(rA)·oligo(rI)-directed poly(dT) synthesis by AMV DNA polymerase. Standard AMV DNA polymerase reaction mixtures (0.3 mL) containing [^{3}H]dTTP as the radiolabeled substrate were prepared as described in Experimental Procedures with the only variation in the template and primer added. All reaction mixtures were incubated at 37 °C, and 50-μL aliquots were removed at the indicated times. The amount of poly(dT) synthesis was measured as picomoles of [^{3}H]dTMP incorporated/0.1 mL of reaction volume with complete 3′-$(rC)_{30-40}$-$(rA)_{\overline{300}}$·$(rI)_{10-20}$ template·primer (●), 3′-$(rC)_{30-40}$-$(rA)_{\overline{300}}$ template alone (○), unmodified $(rA)_{\overline{300}}$ template plus $(rI)_{10-20}$ primer (△), and $(rI)_{10-30}$ primer alone (▲).

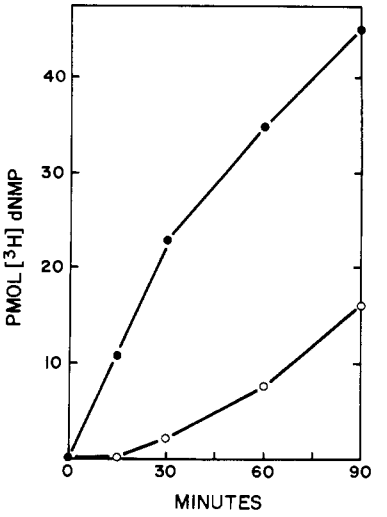

FIGURE 2: Kinetics of 3′-$(rC)_{30-40}$-poly(rA)-oligo(rI)-directed synthesis of poly(dT) and poly(dA) by AMV DNA polymerase. Two standard AMV DNA polymerase reaction mixtures (0.3 mL) were prepared as described in Experimental Procedures containing either [^{3}H]dTTP or [^{3}H]dATP as the radiolabeled substrate and 3′-$(rC)_{30-40}$-$(rA)_{\overline{300}}$·$(rI)_{10-20}$ as the template·primer. Upon incubation of 37 °C, 50-μL aliquots were removed at the indicated times. The amount of poly(dT) and poly(dA) synthesis was measured as picomoles of [^{3}H]dTMP (●) and [^{3}H]dAMP (○) incorporated, respectively, per 0.1 mL of reaction volume.

modified poly(rA) template in the presence or absence of oligo(rI) primer and oligo(rI) primer alone were totally nonfunctional. While 3′-$(rC)_{30-40}$-$(rA)_{\overline{300}}$ template alone did have template·primer activity, it represented only one-sixth the activity of the complete 3′-$(rC)_{30-40}$-$(rA)_{\overline{300}}$·$(rI)_{10-20}$ template·primer. This self-priming activity could have resulted from formation of a hairpin-type structure of the poly(rC) portion at the 3′ end of the RNA template. To determine further if this 3′-modified RNA template·primer was capable of directing the synthesis of dsDNA, namely, poly(dT)·poly(dA), we prepared identical reaction mixtures containing 3′-modified poly(rA)·oligo(rI), purified DNA polymerase, and either [^{3}H]dTTP or [^{3}H]dATP as the radiolabeled substrate to monitor poly(dT) and poly(dA) synthesis, respectively. Figure 2 illustrates the results. Poly(dT) synthesis began immediately upon incubation of the synthetic template·primer with purified AMV DNA polymerase, whereas initiation of poly(dA) synthesis was observed after a brief delay. The lag time for initiation of poly(dA) synthesis with this model template·primer was very dependent on the average length of the poly(rA) template, as would be expected with an RNA template relatively uniform in length and having a specific site for primer binding and initiation of DNA synthesis. When much shorter poly(rA) (average length of 100–150 AMP residues) was modified at its 3′ end and used as template, the lag time was reduced to one-half that observed with the longer poly(rA) template.

Additional features of the template activity of 3′-$(rC)_{30-40}$-$(rA)_{\overline{300}}$ became evident upon examining the substrate requirements for DNA synthesis (Table I). For both poly(dT) and poly(dA) synthesis, dGTP was essential, and dTTP was required for poly(dA) synthesis, suggesting that poly(dT) was directing the synthesis of poly(dA). Therefore, the 3′-modified poly(rA) template with oligo(rI) as primer has the essential properties for directing the sequential synthesis of two complementary strands of DNA, with the additional feature of a specific site for initiation of DNA synthesis.

Optimal Conditions for 3′-$(rC)_{30-40}$-Poly(rA)·Oligo(rI)-Directed DNA Synthesis. The rates of poly(dT) and poly(dA) synthesis on employment of 3′-$(rC)_{30-40}$-$(rA)_{\overline{300}}$·$(rI)_{10-20}$ as

Table I: Substrate Requirements for 3′-$(rC)_{30-40}$-Poly(rA)·Oligo(rI)-Directed Poly(dT) and Poly(dA) Synthesis[a]

reaction conditions	incorporation (pmol)	
	[^{3}H]dTMP	[^{3}H]dAMP
complete	41.4	24.0
-dGTP	0.8	0.9
-dTTP		0.4
-dATP	42.5	

[a] The conditions of a complete reaction mixture containing [^{3}H]dTTP or [^{3}H]dATP as the radiolabeled substrate and 3′-$(rC)_{30-40}$-$(rA)_{\overline{300}}$·$(rI)_{10-20}$ as template·primer are described in Experimental Procedures. Reaction mixtures were incubated for 60 min at 37 °C.

template·primer were dependent upon the concentrations and molar ratios of reaction components. At a fixed concentration of purified AMV DNA polymerase (1 unit), optimal rates of poly(dT) and poly(dA) synthesis were achieved with 3′-modified poly(rA) template at a concentration of 4 μM template AMP residues (Figure 3A). This represents 400 pmol of AMP residues/0.1 mL of reaction volume. By varying the amount of the oligo(rI) primer in annealing mixtures with this concentration (4 μM) of RNA template, the primer–template molar ratio for optimal DNA synthesis was also determined. The results indicate that the rates of both poly(dT) and poly(dA) synthesis were optimal at a primer–template molar ratio of 1:1 (Figure 3B). An increase in the rate of poly(dA) synthesis was the major effect of increasing the concentration of purified DNA polymerase in reaction mixtures. At levels of enzyme greater than 1 unit, the rate of poly(dT) synthesis did not increase significantly, whereas the rate of poly(dA) synthesis was still increasing at a level of 4 units of enzyme. The concentration of dTTP required for optimal poly(dT) synthesis was 0.2 mM and was not noticeably affected by the concentration of dATP in the reaction at suboptimal (0.05 mM) or optimal (0.2 mM) dTTP concentrations. In contrast, the optimal rate of poly(dA) synthesis was affected by the concentration of dTTP present. At 0.2 mM dTTP, an optimal rate of poly(dA) synthesis was

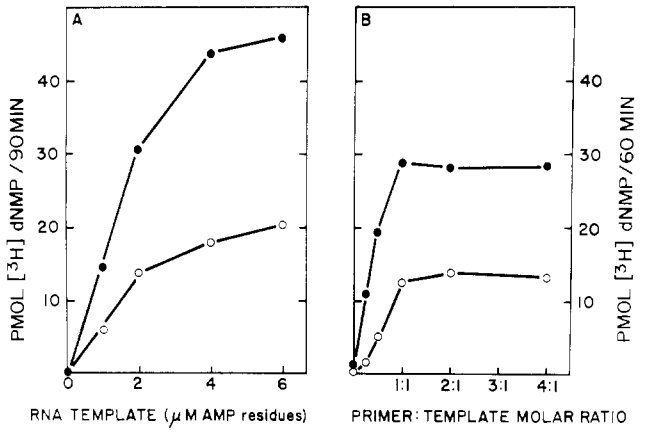

FIGURE 3: Template concentration and primer–template molar ratio for optimal synthesis of poly(dT) and poly(dA) by AMV DNA polymerase. Standard AMV DNA polymerase reaction mixtures (0.1 mL) were prepared as described in Experimental Procedures, containing [^{3}H]dTTP or [^{3}H]dATP as radiolabeled substrate. (A) An increasing amount of previously annealed 3′-$(rC)_{30-40}$-$(rA)_{\overline{300}}$ template·primer (template–primer molar ratio of 1:1) was added to each reaction mixture, and the mixture was incubated at 37 °C for 90 min. The amount of RNA template is indicated as the concentration of AMP residues. (B) A constant amount of 3′-$(rC)_{30-40}$-$(rA)_{\overline{300}}$ template (400 pmol of AMP residues) was preannealed with increasing amounts of $(rI)_{10-20}$ and added to reaction mixtures to achieve the primer–template molar ratios indicated. Reactions were incubated for 60 min at 37 °C. All reaction mixtures were terminated at 0 °C and spotted on DE-81 ion-exchange filter paper disks as previously described. The amounts of poly(dT) and poly(dA) synthesis were measured as picomoles of [^{3}H]dTMP (●) and [^{3}H]dAMP (○) incorporated, respectively.

achieved at 0.1 mM dATP, whereas at the lower dTTP concentration (0.05 mM) more dATP (0.2 mM) was required for an optimal rate of poly(dA) synthesis. While dGTP was a necessary requirement for poly(dT) and poly(dA) synthesis, varying its concentration from 0.05 to 0.2 mM did not affect the rate of either poly(dT) or poly(dA) synthesis.

The effects of the divalent cations Mg^{2+} and Mn^{2+} and the monovalent salt KCl on poly(dT) and poly(dA) synthesis were also examined. The concentration of Mg^{2+} required for an optimal initial rate of poly(dT) synthesis was broad (12–20 mM). However, synthesis terminated after 15 min at these levels of Mg^{2+}. In contrast, at lower concentrations of Mg^{2+} (2–8 mM), the initial rate of synthesis was somewhat less, but it continued for more than 90 min with a resulting increase in the total amount of poly(dT) synthesized. The range of Mg^{2+} concentration for optimal poly(dA) synthesis was essentially the same as that for poly(dT) synthesis with a corresponding early termination of synthesis at the higher divalent ion concentrations and extended kinetics of synthesis at lower concentrations of Mg^{2+}. In testing Mn^{2+} ion as the required divalent cation, we determined a sharp optimal concentration of 0.6 mM for both poly(dT) and poly(dA) synthesis. In addition, there was not the variability in the extent of DNA synthesis with increasing Mn^{2+} ion concentration as observed with Mg^{2+}. Mn^{2+} ion was not an effective substitute for Mg^{2+}, achieving rates and extent of synthesis one-sixth to one-third of those observed for Mg^{2+}. Addition of KCl (up to 90 mM) to reaction mixtures had no stimulatory or inhibitory effect on poly(dT) synthesis, but poly(dA) synthesis was inhibited up to 95% at concentrations greater than 40 mM.

Activation of RNase H Activity. With optimal conditions established for 3′-modified poly(rA)-directed dsDNA synthesis, we then asked whether the DNA polymerase–associated RNase H was activated and, if so, where in this sequence of

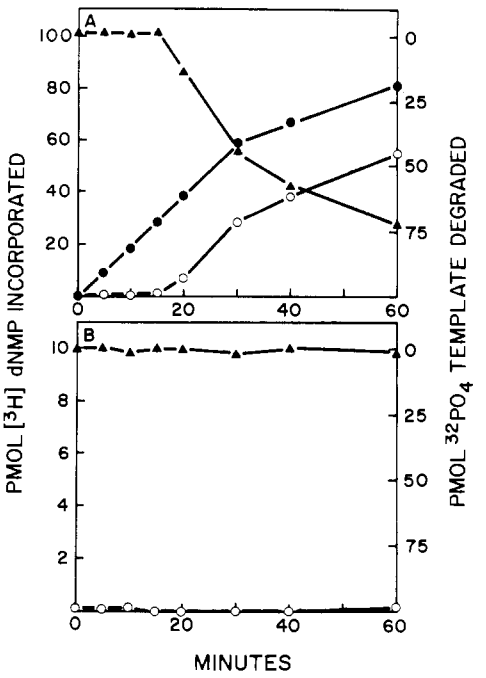

FIGURE 4: Kinetics of AMV DNA polymerase and RNase H activities with 3′-$(rC)_{30-40}$-[^{32}P]poly(rA)·oligo(rI) as template·primer. (A) Two standard AMV DNA polymerase reaction mixtures (0.5 mL) were prepared containing 3′-$(rC)_{30-40}$-[^{32}P]poly(rA)·$(rI)_{10-20}$ template·primer (15 cpm/pmol), 0.2 mM dTTP, dGTP, and dATP, and [^{3}H]dTTP (52 cpm/pmol) or [^{3}H]dATP (36 cpm/pmol) as the radiolabeled substrate. (B) The reaction mixture was identical with that described above except dTTP was omitted and [^{3}H]dATP was the radiolabeled substrate. The synthesis and modification of [^{32}P]poly(rA) for preparation of 3′-$(rC)_{30-40}$-[^{32}P]poly(rA)·$(rI)_{10-20}$ template·primer is described in Experimental Procedures. All reaction mixtures were incubated at 30 °C, and 50-μL aliquots were removed and spotted on DE-81 filter disks at the indicated times. The amounts of poly(dT) and poly(dA) synthesis were measured as picomoles of [^{3}H]dTMP (●) and [^{3}H]dAMP (○) incorporated, respectively, per 0.1 mL of reaction mixture. [^{32}P]Poly(rA) degradation (▲), as measured by release of [^{32}P]poly(rA) from DE-81 ion-exchange paper binding, determined the RNase H activity in the course of DNA synthesis.

synthetic events. Since RNase H degradation of RNA template has been shown to require a RNA·DNA hybrid terminal end (Keller & Crouch, 1972; Leis et al., 1973) and since by design no such hybrid structure was initially present in our template·primer system, it might be expected that RNase H activation would occur at the completion of poly(dT) strand synthesis on the poly(rA) template. Furthermore, if nascent poly(dT) were the template for synthesis of the second strand of DNA as suggested by our data, it might also be the case that synthesis of poly(dA) would begin at or near the time of poly(dT) strand completion, requiring the RNase H activity to free the poly(dT) for further template activity. Thus, activation of RNase H and initiation of poly(dA) synthesis could be closely related events. To test this possibility, we employed a 3′-$(rC)_{30-40}$-[^{32}P]poly(rA)·$(rI)_{10-20}$ template·primer in separate standard reaction mixtures containing [^{3}H]dTTP or [^{3}H]dATP to monitor poly(dT) and poly(dA) synthesis. Because the estimated length (100–150 AMP residues) of the [^{32}P]poly(rA) was one-half to one-third the length of the unlabeled poly(rA) previously described, the reaction mixtures were incubated at 30 °C rather than at 37 °C to slow the synthetic reaction rates, thus maximizing the separation of time-related events. As illustrated in Figure 4A, poly(dT) synthesis was observed first. Degradation of [^{32}P]poly(rA) was not detected before 20 min, at which time initiation of poly(dA) synthesis was also observed. Excluding dATP from the reaction mixtures resulted in no poly(dA) synthesis as

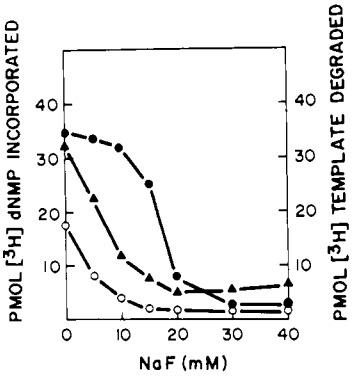

FIGURE 5: Effect of NaF on the synthesis of poly(dT) and poly(dA) and the degradation of [^{3}H]poly(rA) template. Three series of standard AMV DNA polymerase reaction mixtures were incubated containing an increasing concentration of NaF and [^{3}H]dTTP (●) or [^{3}H]dATP (○) as the radiolabeled substrate with unlabeled template·primer or 3′-(rC)$_{30-40}$-[^{3}H]poly(rA)·oligo(rI) (▲) and unlabeled dTTP and dATP. After 30 min at 37 °C, the reaction mixtures were spotted onto DE-81 ion-exchange cellulose filter paper disks and treated for counting as described by Blatti et al. (1970). The specific activities of [^{3}H]dTTP, [^{3}H]dATP, and the [^{3}H]poly(rA) portion of the template were 155, 244, and 16 cpm/pmol, respectively.

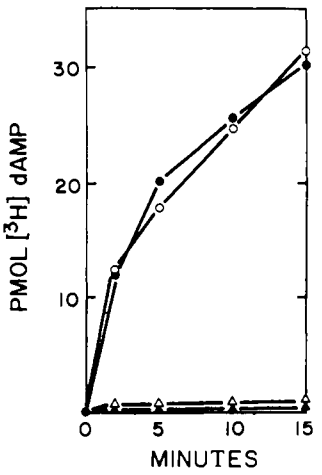

FIGURE 6: Effect of NaF on poly(dT)·oligo(rA)-directed synthesis of poly(dA). Standard AMV DNA polymerase reaction mixtures (0.3 mL) were prepared containing poly(dT)·oligo(rA) template·primer [400 pmol of dTMP residues–20 pmol of rAMP residues, representing a molar ratio of poly(dT)–oligo(rA) of 1:1] and [^{3}H]dATP as the radiolabeled substrate. Upon incubation at 37 °C, 50-μL aliquots were removed, spotted on DE-81 ion-exchange cellulose filter paper disks, and treated for counting as described previously (Blatti et al., 1970). The kinetics of poly(dT)·oligo(rA)-directed poly(dA) synthesis in the presence (○) and absence (●) of 10 mM NaF are shown. (▲) Poly(dA) synthesis directed by poly(dT) alone in the absence of NaF. (△) Poly(dA) synthesis directed by oligo(rA) alone in the absence of NaF.

expected, but both poly(dT) synthesis and poly(rA) degradation proceeded essentially as shown in Figure 4A. When dTTP was omitted from the incubation mixtures, neither synthesis of poly(dT) or poly(dA) nor degradation of [^{32}P]-poly(rA) was observed (Figure 4B). These results not only demonstrate a rather specific activation of RNase H during the model RNA-directed synthesis of DNA but also indicate that initiation of poly(dA) synthesis and activation of RNase H are kinetically related events in the synthesis of dsDNA.

Inhibition of RNase H and Its Effect on dsDNA Synthesis. By use of 3′-(rC)$_{30-40}$-poly(rA)·oligo(rI) as template·primer with purified AMV DNA polymerase, activation of RNase H during DNA synthesis was a delayed event, corresponding in time with the onset of poly(dA) synthesis (Figure 4). This delay of RNase H activation could be simply explained by the delayed synthesis of an appropriate RNA·DNA hybrid substrate and does not in itself indicate a role for RNase H in dsDNA synthesis. However, observation of initiation of poly(dA) synthesis at the same time suggested that RNase H activation may be important in the synthesis of the anti-complementary strand of DNA.

Brewer & Wells (1974) first reported that NaF inhibited the RNase H activity associated with AMV DNA polymerase while it had little or no effect on cDNA synthesis at the same concentration of inhibitor. With our model template·primer system for dsDNA synthesis, we investigated whether the inhibition of RNase H by NaF had any effect on the synthesis of the anticomplementary strand of DNA. Increasing amounts of NaF were added to reaction mixtures containing unlabeled model RNA template·primer and [^{3}H]dTTP or [^{3}H]dATP as the radiolabeled substrates or to reaction mixtures containing 3′-(rC)$_{30-40}$-[^{3}H]poly(rA)·oligo(rI) and unlabeled substrates. After incubation for 30 min, the amounts of synthesis of poly(dT) and poly(dA), as well as poly(rA) degradation, were determined as a function of inhibitor concentration (Figure 5). It can be seen that in the absence of NaF both poly(dT) and poly(dA) were synthesized and the RNase H was actively degrading poly(rA) template. In the presence of 10 mM NaF, the poly(dT) synthesized in 30 min was only slightly less (94% of control) than that synthesized in the absence of NaF. In contrast, the degradation of poly(rA) template and the synthesis of poly(dA) reduced to ca. 37 and 25% of control values, respectively. At higher concentrations of NaF (above 10 mM), poly(dT) synthesis was also inhibited. Therefore, it was only at the lower level of NaF concentration that a selective inhibition of RNase H activity and poly(dA) synthesis was observed.

One explanation for the selective inhibition of poly(dA) synthesis and its relationship to the corresponding inhibition of RNase H activity was that initiation of poly(dA) synthesis was dependent on RNase H activity. If this were the case, inhibition of RNase H activity by NaF would indirectly inhibit poly(dA) synthesis. Alternatively, it was also possible that DNA-directed synthesis of DNA was more sensitive to NaF than RNA-directed synthesis of DNA. To distinguish between these possibilities, we reconstructed a poly(dA) synthesizing reaction that did not require prerequisite poly(dT) synthesis or RNase H activity, yet contained the components most likely present at the onset of poly(dA) synthesis in our model template·primer system. For this, commercial poly(dT) was annealed to oligo(rA) and incubated as template·primer with purified AMV DNA polymerase in the presence and absence of NaF. The results of this experiment are shown in Figure 6 and indicate not only that oligo(rA) adequately serves as primer for poly(dA) synthesis with poly(dT) acting as template but also that the addition of 10 mM NaF to the reaction mixture had no effect on poly(dA) synthesis under these conditions. Therefore, the inhibition of poly(dA) synthesis by NaF (Figure 5) probably was not due to a particular sensitivity of DNA-directed DNA synthesis to NaF inhibition. Rather, the NaF inhibition was specific for RNase H, and poly(dA) synthesis was indirectly affected due to a dependency of dsDNA synthesis on RNase H activity.

Primer for Synthesis of Anticomplementary DNA and the Role of RNase H. Initiation of DNA synthesis by purified AMV DNA polymerase requires an RNA or DNA primer (Kacian et al., 1971; Faras et al., 1972; Leis & Hurwitz, 1972; Taylor et al., 1973). With our 3′-modified poly(rA)·oligo(rI) template·primer model, oligo(rI) acts as primer initiating poly(dT) synthesis at the 3′ end of the RNA template (Figure 1). While poly(dA) synthesis is also observed after a short

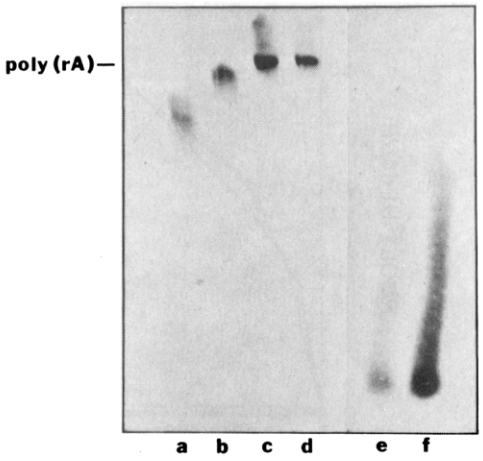

FIGURE 7: Formamide–PAGE analysis of 3′-modified poly(rA)·oligo(rI)-directed poly(dT) and poly(dA) products. [^{3}H]Poly(dT) and [^{3}H]poly(dA) products were prepared in standard reaction mixtures (4.5 and 2.0 mL, respectively) as described in Experimental Procedures containing [^{3}H]dTTP (specific activity, 1700 cpm/pmol) or [^{3}H]dATP (specific activity, 1500 cpm/pmol). Aliquots were removed from each reaction mixture at the times indicated below, phenol-extracted in the presence of 0.5% $NaDodSO_4$ (w/v), and ethanol-precipitated. The samples were resuspended in 0.01 M Tris-HCl, pH 8.8, 0.1 M NaCl, 1 mM EDTA, and 0.1% $NaDodSO_4$ (w/v) and chromatographed on G-50 Sephadex to separate the DNA products from excess radiolabeled substrate. Following alkali treatment in 0.3 N NaOH and 1 mM EDTA for 22 h at 37 °C, the DNA products were concentrated by ethanol precipitation and analyzed by formamide–PAGE and fluorography as described in Experimental Procedures: (a) [^{3}H]-poly(dT) product (98 000 cpm) isolated after 10 min of synthesis; (b) [^{3}H]poly(dT) product (100 000 cpm) after 20 min of synthesis; (c) [^{3}H]poly(dT) product (155 000 cpm) after 60 min of synthesis; (d) [^{3}H]poly(dT) product (115 000 cpm) after 90 min of synthesis; (e) [^{3}H]poly(dA) product (21 000 cpm) after 60 min of synthesis; and (f) [^{3}H]poly(dA) product (64 000 cpm) after 90 min of synthesis.

time lag, the primer for its initiation is not known. Upon further review of our model system and the results just described, at least two possible mechanisms emerge. First, poly(rA)-directed poly(dT) product could act not only as the template for poly(dA) synthesis but also as the primer by assuming a hairpin-like structure at its 3′-OH terminus following degradation of the poly(rA) template from the newly formed poly(rA)·poly(dT) hybrid. Thus, the observed requirement for RNAse H in the synthesis of poly(dA) would be satisfied. Alternatively, poly(rA) fragments generated by RNase H at the 5′ end of the poly(rA) template following RNA·DNA hybrid formation could, in turn, serve as complementary primers for initiation of poly(dA) synthesis by using the nascent poly(dT) as template. This mechanism would also satisfy the observed requirement for RNase H activation in the synthesis of poly(dA). To distinguish between these two mechanisms, we first examined DNA products by formamide–PAGE that were synthesized at various times in the reaction sequence in the presence of [^{3}H]dTTP or [^{3}H]dATP. If nascent poly(dT) were acting as template and primer, a covalent linkage between poly(dT) and poly(dA) should result and [^{3}H]poly(dA) would migrate with poly(dT) during formamide–PAGE. If poly(rA) fragments were acting as primers for initiation of poly(dA) synthesis, a poly(dT)–poly(dA) linkage would not be predicted. Thus, poly(dA) might have a quite different electrophoretic migration pattern. To investigate these possibilities, we isolated [^{3}H]poly(dT) product after 10, 20, 60, and 90 min of synthesis, while [^{3}H]poly(dA) product was obtained after 60 and 90 min. Following phenol extraction, removal of excess radiolabeled substrates, and allkali treatment to hydrolyze remaining RNA template, the DNA products were analyzed with the results

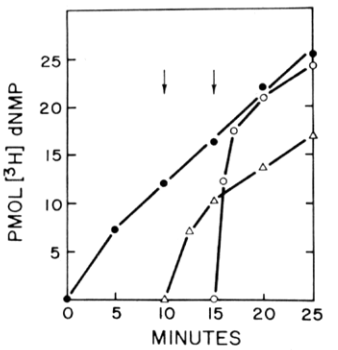

FIGURE 8: Effect of delayed addition of dATP on 3′-modified poly(rA)·oligo(rI)-directed poly(dA) synthesis. Three standard AMV DNA polymerase reaction mixtures (0.3 mL) were prepared with 3′-$(rC)_{30-40}$-$(rA)_{300}$·$(rI)_{10-20}$ as the template·primer. To the first reaction mixture was added [^{3}H]dTTP as the radiolabeled substrate, and upon incubation at 37 °C 50-μL aliquots were removed at 5-min intervals and analyzed for poly(dT) synthesis as previously described (Blatti et al., 1970). [^{3}H]dATP substrate was withheld from the second and third reaction mixtures until 10 (△) and 15 min (○) after incubation at 37 °C. Aliquots of 50 μL of these mixtures were then removed at 5-min intervals for analysis of poly(dA) synthesis.

shown in Figure 7. The size of poly(dT) product increased with time of synthesis, approaching the length of the poly(rA) template by 60 min. In contrast, the major portion of poly(dA) product even after 90 min of synthesis was quite (~60–70 nucleotides in length) and was not migrating with the poly(dT). From these results it seems clear that poly(dT) was not acting in a dual role as the primer and template for synthesis of poly(dA). However, it could not be concluded at this point that poly(rA) fragments resulting from RNase H action were the primers.

Conclusive proof that the poly(rA) fragments resulting from RNase H hydrolysis were acting as primers for initiation of poly(dA) synthesis could most simply be demonstrated by the identification of poly(dA) product in covalent linkage with AMP residues [or poly(rA)]. As formation of this specific linkage would be expected to be a minor event compared to the number of dAMP–dAMP linkages formed and since the RNA primer could be further degraded by RNase H during poly(dA) synthesis, we first examined the possibility of optimizing the formation of RNA–DNA linkages in the synthetic reaction by delaying the addition of dATP to the reaction mixture. In effect, poly(dT) synthesis and poly(rA) degradation were allowed to proceed, setting the stage for initiation of poly(dA) synthesis; but without the dATP no initiation of poly(dA) synthesis was possible (Table I). Separate reaction mixtures were prepared containing 3′-$(rC)_{30-40}$-poly(rA)·oligo(rI), dGTP, [^{3}H]dTTP, and purified AMV DNA polymerase. To one mixture was added [α-^{32}P]dATP after 10 min of incubation, while to the other mixture the delay of addition of [α-^{32}P]dATP was 15 min. The results are shown in Figure 8. Poly(dT) synthesis began upon incubation at 37 °C and proceeded at a near linear rate for more than 25 min. Upon addition of dATP to both reaction mixtures, there was immediate incorporation of [α-^{32}P]dAMP into poly(dA). However, the rate and extent of poly(dA) synthesis were greatest when addition of dATP was delayed for 15 min. Addition of dATP at even later times during the reaction when poly(dT) synthesis had essentially terminated resulted in less efficient poly(dA) synthesis. Therefore, by controlling the time of addition of dATP, it was possible to increase the rate of poly(dA) synthesis and thus increase the number of initiation events in a given time period.

Finally, to demonstrate the presence of rAMP–dAMP covalent linkages in newly synthesized poly(dA), we syn-

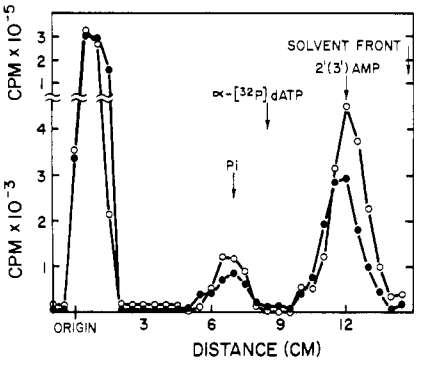

FIGURE 9: Radiochromatogram of degradation product after alkali treatment of [^{32}P]poly(dA). ^{32}P-Labeled poly(dA) was synthesized as described in Experimental Procedures by using purified AMV DNA polymerase, [α-^{32}P]dATP, and 3'-(rC)$_{30-40}$(rA)$_{300}$·(rI)$_{10-20}$ template·primer (●) or poly(dT)·(rA)$_{12-20}$ template·primer (○). Following purification and alkali treatment, an aliquot of each [^{32}P]poly(dA) product (3×10^5 cpm) was analyzed by ascending paper chromatography as described in Experimental Procedures. $^{32}P_i$, 2'(3')-AMP, and [α-^{32}P]dATP were included as controls, and their locations on the chromatogram are so indicated.

thesized poly(dA) product in the presence of [α-^{32}P]dATP under the optimal initiation conditions just described (Figure 8). Following purification to remove protein and unreacted [α-^{32}P]dATP, the DNA product was alkali-treated and the hydrolysate was chromatographed by using an ascending paper chromatography system appropriate for detection of 2'(3')-AMP. In addition to the large amount of [^{32}P]poly(dA) found at the origin of the chromatogram, a peak of radioactivity representing 1.7% of the total cpm applied migrated with the same R_f as the principle product of a [^{3}H]poly(rA) alkaline hydrolysate and 2'(3')-AMP (Figure 9). This frequency of transfer represented 1 rAMP–dAMP linkage/60 dAMP residues in phospho diester linkage, agreeing very well with the estimated size of poly(dA) by formamide–PAGE (Figure 7). As a positive control of the technique, the alkali-treated [^{32}P]poly(dA) product of a poly(dT)·oligo(rA)-directed AMV DNA polymerase reaction was also run on the same chromatogram. Since oligo(rA) was the primer in this synthetic template·primer system, rAMP–dAMP covalent linkages and the appearance of 2'(3')-[^{32}P]AMP on the chromatogram were expected. The results shown in Figure 9 indicate that the degradation products from alkali treatment of poly(dT)·oligo(rA)-directed poly(dA) were identical with those obtained with the 3'-modified poly(rA)·oligo(rI) template·primer system, including 2'(3')-AMP and, for unknown reasons, a small amount of radioactive P_i (Wells et al., 1972). Therefore, we have demonstrated the synthesis of rAMP–dAMP covalent linkages during poly(dA) synthesis. Since poly(dA) synthesis is not observed in the absence of RNase H activity and RNase H degrades the poly(rA) template (Figure 4), the data further indicate that poly(rA) species smaller than intact poly(rA) template are acting as primers, and RNase H is responsible for their production.

Discussion

The synthetic ribohomopolymer poly(rA) has been previously demonstrated as a useful model RNA template for elucidating the processes of initiation and elongation of RNA-directed cDNA synthesis by RNA tumor virus DNA polymerases (Baltimore & Smoler, 1971; Goodman & Spiegelman, 1971; Smoler et al., 1971; Leis & Hurwitz, 1972). In the presence of oligo(dT) or oligo(rU) as primer, it directs the synthesis of poly(dT). In this study, we have investigated whether this model RNA template might also be useful for examining the role of purified AMV DNA polymerase and its associated RNase H activity in the synthesis of RNA-directed dsDNA. Initially, it was necessary to better define and control the earliest event of DNA synthesis, namely, the initiation of poly(dT) synthesis. This was accomplished by modification of the poly(rA) at its 3'-terminal end. Addition of CMP residues by using *M. luteus* polynucleotide phosphorylase offered the possibility of initiation of poly(dT) synthesis at a specific site on the RNA template by using oligo(rI) primer. Therefore, unlike the poly(rA)·oligo(dT) or poly(rA)·oligo(rU) template·primers in which multiple primer sites for initiation were possible, initiation of DNA synthesis with this model was a specific event at the 3' terminus. Furthermore, on employment of a sized poly(rA) template, the events leading to dsDNA synthesis were likely to occur more uniformly. Our results indicate that, first, oligo(rI) primer that was previously annealed to the 3'-poly(rC) portion of the poly(rA) template was extended in the presence of dGTP and dTTP as substrates. That poly(dT) synthesis was totally dependent on the presence of dGTP indicated the specificity of the priming event. Also, it suggested that a large portion of the oligo(rI) primer must have annealed to the 3'-poly(rC) tail at a position on the 3' side of the CMP–AMP linkage rather than directly at the position of the CMP–AMP linkage.

Following initiation of poly(dT) synthesis, there was a definite time lag before initiation of poly(dA) synthesis. Also, dGTP and dTTP, as well as dATP, were required as substrates. This suggested strongly that poly(dT) synthesis was required for poly(dA) synthesis and that poly(dT) was probably acting as template directing the synthesis of poly(dA). In addition, these results demonstrated that purified AMV DNA polymerase, in the presence of the 3'-modified poly(rA)·oligo(rI) template·primer, was capable of directing the synthesis of two complementary strands of DNA from a specific initial primer point.

Previous studies on the characterization of retrovirus DNA polymerase-associated RNase H have for the most part been carried out in the absence of RNA-directed DNA synthesis. Consequently, while various functions have been proposed for RNase H during reverse transcription (Moelling et al., 1971; Baltimore & Smoler, 1971; Keller & Crouch, 1972; Leis et al., 1973; Collett & Faras, 1978; Myers, & Spiegelman, 1978), its precise role in RNA-directed DNA synthesis has remained obscure. To examine its function(s) during active DNA synthesis, we employed our specific model RNA template·primer [3'-(rC)$_{30-40}$-poly(rA)·oligo(rI)] containing radiolabeled poly(rA). Immediately upon incubation at 37 °C in the presence of purified AMV DNA polymerase and the appropriate deoxyribonucleoside triphosphates, this RNA template·primer directed the synthesis of poly(dT). After a brief lag time, presumably to complete the reverse transcription of the 3'-modified poly(rA) template to its 5' terminus, RNase H was activated and poly(rA) template was degraded. Therefore, the first obvious action of RNase H in this synthetic system was nucleolytic degradation of the poly(rA) template upon formation of a RNA·DNA hybrid terminus. However, at about the same time of RNase H activation, poly(dA) synthesis was also initiated. This led us to consider whether RNase H was required for synthesis of the anticomplementary strand of DNA. By use of NaF, a specific inhibitor of RNase H (Brewer & Wells, 1974), and a reconstructed model template·primer [poly(dT)·oligo(rA)] that was most likely responsible for poly(dA) synthesis in our 3'-modified poly(rA) template system, it was shown that the DNA polymerase–

associated RNase H activity was required for initiation of synthesis of the anticomplementary strand of DNA.

The function of RNase H in the synthesis of the anticomplementary strand of DNA with this model template·primer system could be that of freeing the nascent poly(dT) from the poly(rA)·poly(dT) hybrid so that poly(dT) could further act as DNA template for synthesis of poly(dA). However, it was also necessary to consider that AMV DNA polymerase required a primer as well as a template for initiation of poly(dA) synthesis. The properties of our model RNA template·primer suggested that either poly(dT) was acting as template and primer by a hairpin–loop mechanism or the RNase H generated poly(rA) fragments were functioning as primers. That poly(dT) was not acting as both template and primer for poly(dA) synthesis was demonstrated by the absence of poly(dT)–poly(dA) covalent linkages in the DNA product. Therefore, simply freeing nascent poly(dT) by RNase H action was not a sufficient explanation for the onset of poly(dA) synthesis.

Direct evidence that the template poly(rA) fragments were acting as primers for synthesis of poly(dT)-directed poly(dA) was obtained by identifying the presence of rAMP–dAMP phosphodiester linkages in the DNA product. Since the only other possible RNA–DNA covalent linkages present would have been rIMP–dGMP and rIMP–dTMP, and neither [α-^{32}P]dGTP nor [α-^{32}P]dTTP was used as radiolabeled substrate, transfer of PO_4 to 2′(3′)-rIMP would not have been detectable. It has been well documented that retrovirus DNA polymerases including AMV DNA polymerase utilize both natural and synthetic homopolymeric RNA primers for initiation of RNA-directed cDNA synthesis [for reviews, see Green & Gerard (1974) and Taylor (1977)]. However, to the present time, the primer for initiation of the anticomplementary strand of the DNA during dsDNA synthesis has not been identified. From the studies described here in which a synthetic model RNA template·primer was employed, an RNA species is also acting as the primer for synthesis of the second strand of DNA. Furthermore, the activity of AMV DNA polymerase–associated RNase H plays an important role in generating these RNA primer molecules by RNA template degradation. The possibility that these observations and assigned functions are relevant to the role of the AMV DNA polymerase complex in retrovirus RNA-directed synthesis of dsDNA is also significant. Current studies in our laboratory suggest a role for RNase H in viral 35S RNA-directed dsDNA synthesis very similar to that described for the model system. Reverse transcription of the viral RNA at its 3′ terminus initially forms a RNA·DNA hybrid that acts as substrate for RNase H. Almost immediately upon RNase H degradation of RNA template, dsDNA is observed and the newly synthesized anticomplementary strands of DNA contain RNA–DNA covalent linkages (unpublished experiments).

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Reversible Modification of *Escherichia coli* Ribosomes with 2,3-Dimethylmaleic Anhydride. A New Method to Obtain Protein-Deficient Ribosomal Particles†

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ABSTRACT: Treatment of *Escherichia coli* ribosomes with the protein reagent 2,3-dimethylmaleic anhydride is accompanied by inactivation of polypeptide polymerization and by dissociation of ribosomal proteins. Regeneration of the modified amino groups at pH 6.0 is followed by reactivation and reconstitution of the ribosomes. Prior to regeneration of the amino groups, ribosomal particles and split proteins can be separated by centrifugation, which allows the preparation of new protein-deficient particles. The ribosomal particles obtained by three successive treatments with 2,3-dimethylmaleic anhydride at a molar ratio of reagent to ribosome equal to 16 000 lack proteins S1, S2, S3, S5, S10, S13, S14, L7, L8, L10, L11, L12, and L20 and have lost part of proteins S4, L1, L6, L16, and L25. This new procedure to obtain protein-deficient ribosomal particles is mild and might be useful to dissociate other protein-containing structures in addition to ribosomes.

The protein reagents succinic anhydride, maleic anhydride, and DMMA[1] modify amino groups substituting a negative charge for a positive one, which brings about a drastic change in electrostatic properties frequently accompanied by dissociation of proteins into their subunits (Means & Feeney, 1971; Klotz & Keresztes-Nagy, 1962; Freisheim et al., 1967). Amino groups modified by DMMA can be easily regenerated at moderate low values of pH (Dixon & Perham, 1968).

Modification of *Escherichia coli* ribosomes by succinic, maleic, and acetic anhydrides is followed by inactivation of polypeptide polymerization, peptidyl transferase, and elongation factor G-dependent GTPase, dissociation of the 70S ribosomes into 50S and 30S subunits, and, probably, separation of individual proteins (Pintor-Toro et al., 1978). Since DMMA can be easily removed with regeneration of the modified amino groups, this reagent might be used to dissociate proteins from the ribosome and to obtain the corresponding protein-deficient particles.

The present paper reports a new method to prepare ribosomal "cores" and split proteins based on the reversible modification of protein amino groups with the reagent DMMA, which causes dissociation of ribosomal proteins in a fairly specific way.

Materials and Methods

Preparation of Ribosomes and Assay of Activities. Ribosomes were prepared from *E. coli* MRE 600 by grinding the cells with alumina, followed by differential centrifugation. Ribosomes were washed three times with 1 M NH_4Cl, suspended in 5 mM Tris-HCl (pH 7.8), 20 mM magnesium acetate, 500 mM NH_4Cl, 2 mM dithiothreitol, 0.5 mM EDTA and 50% (v/v) glycerol, at a ribosomal concentration of 75 mg/mL, and kept at −20 °C (Modolell & Vázquez, 1973). The 30S subunits were prepared by zonal centrifugation (Eikenberry et al., 1970). Poly(U)-directed polyphenylalanine synthesis was determined in a crude system containing S-100 extract (Nirenberg & Matthaei, 1961). Peptidyl transferase was estimated by the "fragment reaction" assay, using C-(U)-A-C-C-A-(Ac[^{3}H]Leu) and puromycin as substrates (Monro, 1971).

Modification of Ribosomes, Separation of Ribosomal Particles from Split Proteins, and Regeneration of the Modified Groups. Prior to treatment with DMMA, 0.2 mL of stored ribosomes (15 mg) was diluted with 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (K^+) (Hepes) (pH 8.2), 20 mM magnesium acetate, 0.5 mM dithiothreitol, and 0.5 mM EDTA, to a final volume of 2 mL. To this solution, the reagent (300 mg/mL of DMMA in dioxane) was added stepwise in aliquots of 5 μL, and the pH was maintained at 8.2 by addition of 0.5 M KOH. The treatment took place at room temperature and was complete in 1 h. To separate the ribosomal particles from the split proteins, immediately after DMMA treatment the volume of the preparation was increased to 5 mL with 50 mM Tris-HCl (pH 8.2), 20 mM magnesium acetate, 50 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM EDTA and centrifuged for 4.5 h at 2 °C and 64 000 rpm in a Beckman SW65 rotor. After centrifugation, the supernatant was collected and the sediment suspended in 2 mL of the above mentioned solution. To regenerate the modified amino groups, the corresponding preparation was dialyzed for 48 h at 0–5 °C against 20 mM

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[1] Abbreviation used: DMMA, 2,3-dimethylmaleic anhydride.

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