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RNA-Dependent DNA Polymerase Activity of RNA Tumor Virus

VI. Processive Mode of Action of Avian Myeloblastosis Virus Polymerase

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Purified avian myeloblastosis virus (AMV) polymerase consisting of α,β subunits has been shown to act processively in catalyzing DNA synthesis primed with 34S AMV RNA·oligo(dT), poly(A)·poly(dT), and poly(I)·poly(dC). DNA transcripts prepared with 34S AMV RNA·oligo$(dT)_{14}$ and AMV polymerase ($\alpha\beta$) have been shown to have a molecular weight of 1.05×10^6, or approximately one-third the size of the 34S RNA genome. Polymerase subunit α acts nonprocessively with the above templates.

RNA-dependent DNA polymerases have been purified from several different oncornaviruses (1, 3, 5, 6, 10, 13, 17, 18, 24, 26, 29, 30) and are all similar in that they catalyze repair-like DNA synthesis on RNA, DNA, and RNA·DNA hybrids. The DNA transcripts synthesized are small, sedimenting as 6-7S material when measured by alkaline sucrose gradient centrifugation. Reverse transcriptase, purified from avian myeloblastosis virus (AMV), has been reported to consist of two polypeptides corresponding to molecular weights of 68,000 (α) and 105,000 (β) determined by sodium dodecyl sulfate-gel electrophoresis (13). The major form of the enzyme isolated from virus is a complex of $\alpha\beta$ in a 1:1 molar ratio. Small amounts of catalytically active α subunits elute from phosphocellulose at lower ionic strengths than the $\alpha\beta$ enzyme complex. Purified β subunits have not been isolated from AMV, although preparations of catalytically active β subunits have been isolated from B77 by A. Hizi and W. K. Joklik (personal communication).

Gibson and Verma (7) have shown that the amino acid sequence of the α subunit is closely related to the β subunit since all iodine-labeled, trypsin-resistant polypeptides prepared from the α subunit correspond to polypeptides prepared from β. In agreement with these results, Moelling (19) has presented data suggesting that the α subunit is derived from the β subunit after proteolytic cleavage.

Most RNA-dependent DNA polymerases studied so far have RNase H activity (8, 14, 15, 21, 28) detectable in both α and $\alpha\beta$ forms of the enzyme. The RNase H associated with $\alpha\beta$ enzyme is a processive exonuclease that can cleave RNA in hybrid structure bidirectionally (15). In contrast, the RNase H activity associated with α is nonprocessive in action (9).

We have examined the mode of action of the AMV polymerase DNA synthesis in this report and have found that the $\alpha\beta$ form of the enzyme acts processively, whereas the α form appears to act nonprocessively. This result suggests that the polypeptide fragment cleaved from the β subunit to form the α subunit is involved in the binding of the polymerase to polynucleotides. The multimeric form of polymerase ($\alpha\beta$), at high enzyme/template ratios, is capable of transcribing only one-third of the length of 34S RNA into RNA when synthesis is initiated from oligo$(dT)_{14}$ annealed to the 3'-terminal poly(A) sequence of 34S RNA.

MATERIALS AND METHODS

The labeled deoxynucleoside triphosphates were commercial preparations from New England Nuclear Corp. Calf thymus DNA was obtained from Worthington Biochemicals Corp. Synthetic homopolymers and oligo$(dT)_{14}$ were from Biopolymers or P-L Biochemical Corp. Dithioerythritol and actinomycin D were from Sigma Chemical Co. ^{3}H-labeled adeno-associated virus DNA was a gift of John Newbold, University of North Carolina, Chapel Hill. AMV was a generous gift of Dani Bolognesi and Alphonse Langlois (this institution) and Joseph Beard, Life Sciences, St. Petersburg, Fla.

Purification of AMV polymerase. The AMV polymerase was purified from 1,500 ml of citrate-treated plasma derived from leukemic chickens (7×10^{11} virus particle/ml) by a modification of the procedure described by Leis and Hurwitz (16). Polymerase activity was measured by incorporation of

[^{3}H]dTTP (50 cpm/pmol) into acid-insoluble form after 10 min of incubation at 38°C in a reaction primed with poly(A) (6.4 nmol)·oligo(dT)$_{14}$ (0.96 nmol). A unit of polymerase is defined below. The polymerase was eluted from a phosphocellulose column (60-ml column volume, 1.5 by 29 cm) with a 600-ml linear gradient from 0 to 0.5 M ammonium sulfate in 10 mM Tris-hydrochloride, pH 8.0, 0.1 mM EDTA, 2 mM dithioerythritol, and 10% glycerol. Fractions (5 ml) were collected. Enough 10% Triton X-100 was placed in the collection tubes to bring the final concentration to 0.1%. The polymerase fraction $\alpha\beta$ eluting at 0.19 M ammonium sulfate (which contained 98% of the polymerase activity bound to the column, 15,000 units in a 40-ml volume) was collected and concentrated on a Schleicher & Schuell protein concentrator with a no. 100 collodion bag for 48 h at 4°C against a buffer containing 10% glycerol, 0.2 M ammonium sulfate, 0.1 mM EDTA, 0.1% Triton X-100, 10 mM Tris-hydrochloride, pH 8, and 4 mM dithioerythritol. The enzyme was concentrated to a volume of 0.7 ml (11,300 units) by this procedure. An additional 3,500 units were recovered by washing the dialysis bag with 0.6 ml of the above buffer. Polymerase subunit α (300 units in a volume of 23 ml), which eluted from phosphocellulose at 0.08 M ammonium sulfate, was treated in a similar fashion. Polymerase $\alpha\beta$ was layered directly onto a 10-ml 15 to 40% linear glycerol gradient containing 0.2 M ammonium sulfate, 0.1% Triton X-100, 0.1 mM EDTA, 10 mM Tris-hydrochloride, pH 8, and 4 mM dithioerythritol and centrifuged in a Spinco centrifuge in an SW41 rotor at 40,000 rpm for 64 h at 4°C. Thirty-drop fractions were collected from a hole pierced in the bottom of the tubes, and samples were assayed for polymerase activity. The polymerase ($\alpha\beta$) migrated approximately 78% down the gradient, and recovery of activity was greater than 90%. The polymerase ($\alpha\beta$) is free of detectable contaminating RNase (with the exception of RNase H) and DNase activities as previously described (10, 15, 17). Polymerase α is free of detectable DNase and most RNase activity. A limited amount of endonuclease could still be detected by incubating 2 units of polymerase with ^{32}P-labeled 34S Rous sarcoma virus RNA (65 cpm/pmol, 50 pmol) for 60 min at 38°C. Analysis of the ^{32}P-labeled 34S Rous sarcoma virus RNA on sodium dodecyl sulfate-sucrose gradients after heating at 80°C for 2 min showed one to two breaks per strand.

Isolation of 34S AMV RNA. AMV was collected from fresh unfrozen plasma from leukemic chickens by centrifugation in a Spinco 35 rotor at 22,000 rpm for 35 min at 4°C. Virus pellets were rinsed with 0.1 M NaCl, 10 mM Tris-hydrochloride, pH 7.5, and 1 mM EDTA, suspended in the above buffer modified to contain 1% sodium dodecyl sulfate, and extracted three times with an equal volume of phenol (saturated with water). RNA was collected from the aqueous phase after precipitation with 2 volumes of 95% ethanol. The 60 to 70S RNA was purified by zone sedimentation in neutral sucrose gradients. The material sedimenting in the region between 60-70S, which was followed by measuring its absorbance at 260 nm in a Gilford spectrophotometer, was precipitated with alcohol and collected by centrifugation in a Spinco SW50.1 rotor at 49,000 rpm for 60 min at 4°C. Three to four absorbancy units at 260 nm of 60S RNA was routinely isolated from the virus contained in 300 to 500 ml of plasma. Virus 60S or 34S RNAs (prepared by heating 60S RNA at 80°C for 2 min) were found to be contaminated with RNase, which could be detected by incubating the RNA at 30°C for 10 min in the presence or absence of $MgCl_2$ followed by sucrose gradient analysis. The 34S RNA free of RNAse was prepared by suspending the ethanol-precipitated 60S RNA in 0.1 ml of a buffer containing 0.1 M NaCl, 10 mM Tris-hydrochloride, pH 7.5, and 1 mM EDTA and layering it onto a 5-ml linear gradient of 5 to 20% sucrose in 80% dimethyl sulfoxide (Me_2SO), 10 mM LiCl, 10 mM Tris-hydrochloride, pH 7.5, 1.0 mM EDTA. Gradients were centrifuged in a Spinco SW50.1 rotor at 49,000 rpm for 22 h at 20°C. The 34S RNA, found approximately 65 to 80% down the gradient, was precipitated with 2 volumes of 95% ethanol; the mixture was stored at −20°C overnight and collected as described above. The 34S RNA prepared by the above procedure was free of detectable RNase activity and could be incubated for 60 min at 37°C without any observed change in its sedimentation properties after heat denaturation. In addition, all small RNA molecules associated with 34S RNA were removed during the centrifugation procedure.

Preparation of 34S RNA·oligo(dT)$_{14}$. Oligo(dT)$_{14}$ was annealed to 34S RNA (suspended in 0.1 M NaCl, 10 mM Tris-hydrochloride, pH 7.5) by successive 2-min incubations at 45, 37, and 30°C. Concentrations of RNA and oligo(dT)$_{14}$ are indicated in the figure legends.

Preparation of poly(A)·poly(dT) and poly(I)·poly(dC). Homopolymeric pairs were annealed as above except polymers were melted at 58°C and cooled slowly. Concentrations are as indicated in the figure legends.

Measurement of polymerase activity. Polymerase activity was measured as follows. Reaction mixtures (0.05 ml) contained 1 μmol of Tris-hydrochloride, pH 8.0, 0.1 μmol of dithioerythritol, 2 μmol of KCl, 0.5 μmol of $MgCl_2$, 2.2 μg of albumin, 25 nmol each of dATP, dTTP, and dGTP, labeled dCTP (specific activity and concentration as indicated), and 34S RNA·oligo(dT)$_{14}$ and enzyme as indicated. Actinomycin D or ATP was added to the incubation where indicated. After 10 min at 30°C, the reaction was terminated by the addition of 0.1 ml of 0.1 M sodium pyrophosphate, 0.02 ml of denatured salmon sperm DNA (2.4 μmol/ml), and 5% trichloroacetic acid. Acid-insoluble material was collected on Gelman type E glass-fiber filters, dried, and counted in a liquid scintillation counter. One unit of polymerase is defined as the amount of enzyme that incorporates 1 nmol of nucleotide in 30 min under the above conditions.

Size of DNA products measured by alkaline sucrose gradient centrifugation. The size of the DNA transcripts was analyzed as follows. Polymerase reactions were terminated with 5 μmol of EDTA, and protein was extracted with an equal volume of phenol (0.05 ml). The phenol phase was removed

with a micropipette, and the aqueous phase was dialyzed for 2 h at 4°C against 500 ml of a solution containing 1× SSC (SSC = 0.15 M NaCl + 0.015 M sodium citrate). Recovery of DNA varied between 60 and 80%. RNA was removed from the dialyzed DNA samples by adding 10 N NaOH to a final concentration of 0.3 M, followed by incubation at 25°C for 3 h. Under these conditions ^{32}P-labeled 34S Rous sarcoma virus RNA (60 cpm/pmol) was rendered 98% acid soluble. Samples were then layered onto 5 to 20% linear sucrose gradients in 0.3 N NaOH, 0.7 M NaCl, and 5 mM EDTA and centrifuged at 49,000 rpm in a Spinco SW50.1 rotor at 20°C for various lengths of time (25). Fractions were collected from a hole pierced in the bottom of the tube, and the acid-insoluble radioactivity was determined. Recovery of label from gradients was greater than 80%. Molecular weight was calculated by the method of Studier (25), using circular ϕX DNA (1.7×10^6) and linear adeno-associated virus DNA (1.4×10^6) as reference markers.

RESULTS

Processive mode of action of AMV polymerase ($\alpha\beta$) on synthetic homopolymers. A processive polymerase is one that binds to a primer-template and catalyzes the polymerization of a large number of nucleotides before it is released. A nonprocessive polymerase is released from the primer-template after polymerization of one or a limited number of nucleotides. The AMV polymerase ($\alpha\beta$) was tested for its mode of action by measuring the initial rates of synthesis of [^{3}H]poly(dT) or [^{32}P]poly(dC) in reactions primed with poly(A)·poly(dT) and poly(I)·poly(dC), respectively, under conditions in which the amount of enzyme was limiting. Increasing the concentration of template by as much as sixfold did not alter the initial rate of deoxynucleotide incorporation, whereas doubling the amount of polymerase in the incubation resulted in twice the observed incorporation. The initial rates of poly(dT) and poly(dC) synthesis were linear from zero time to the end of the measured reaction (Fig. 1A). When both primer-templates were mixed together in equal amounts before addition of the enzyme, the rate of synthesis of either polymer was less than the rate observed with each primer-template alone (Fig. 1B). When poly(dC) synthesis was initiated first on poly(I)·poly(dC) and then an equal amount of poly(A)·poly(dT) was added 1 min after the start of the reaction, the rate of poly(dC) synthesis was unaffected. There was no detectable poly(dT) synthesis until several minutes after addition of the poly(A)·poly(dT) (Fig. 1C). It is possible that the above result can be explained if the polymerase has a different K_m for binding each of the homopolymers. For this reason this experiment was repeated reversing the order of addition (Fig. 1D), and identical results were obtained.

Processive mode of action of AMV polymerase ($\alpha\beta$) on 34S AMV RNA·oligo(dT). These studies were extended to 34S AMV RNA. Oligo(dT)$_{14}$ was annealed to 34S AMV RNA as a primer, since 34S RNA isolated from Me_2SO gradients lacked small RNA primer molecules. Wang and Duesberg (31) have shown that poly(A) sequences are found solely at the 3′ terminus. Thus, oligo(dT)$_{14}$ annealed to the poly(A) region provides a primer at the 3′ end for DNA synthesis to proceed in the 3′ to 5′ direction with the entire length of the 34S RNA molecule available as template. This is particularly important since the tRNA$_{trp}$ primer, from which most DNA is initiated on 60S RNA in vitro, has been shown by us (manuscript in preparation) as well as by Taylor and Illmensee (27) to be located near the 5′ terminus. Under the conditions of the assay (30°C), the DNA synthesis observed represented transcription of 34S RNA rather than specific poly(dT) synthesis since: (i) the molar incorporation of α-[^{32}P]dCTP was the same as [^{3}H]dTTP; (ii) alkaline sucrose gradient analysis of the DNA transcripts formed in the above reaction showed that the ratio of ^{3}H to ^{32}P was constant for all DNA sizes; and (iii) oligo(dT)$_{14}$-stimulated DNA synthesis was dependent on annealing oligo(dT)$_{14}$ to 34S RNA prior to the reaction.

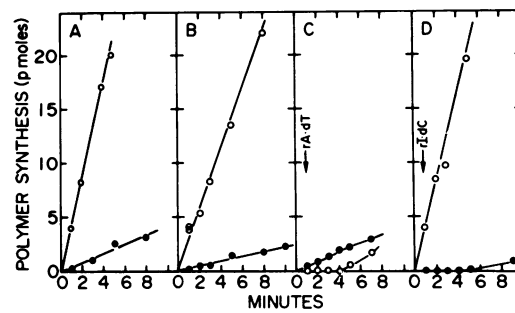

FIG. 1. *Processive mode of action of AMV polymerase on synthetic homopolymers. Reaction mixtures (0.05 ml) containing AMV polymerase ($\alpha\beta$, 0.46 unit) were incubated as described in the text with [^{3}H]dTTP (820 cpm/pmol, 5.75 nmol), α-[^{32}P]dCTP (1,389 cpm/pmol, 4.9 nmol), poly(A) (1.2 nmol)·poly(dT) (0.25 nmol), and/or poly(I) (1.09 nmol)·poly(dC) (0.32 nmol) as indicated. After incubation at 30°C as indicated, acid-insoluble radioactivity was determined. (A) Each polymer pair was incubated with polymerase separately; (B) both polymer pairs were incubated with polymerase together; (C) enzyme was incubated with poly(I)·poly(dC) for 1 min at which time poly(A)·poly(dT) was added; (D) enzyme was incubated with poly(A)·poly(dT) and poly(I)·poly(dC) was added 1 min after the start of the reaction. Symbols: poly(dT) synthesis, ○; poly(dC) synthesis, ●.*

DNA synthesis primed with 34S RNA·oligo(dT)$_{14}$ was assayed by following the incorporation of [^{3}H]dTTP into acid-insoluble form in the presence of the other three deoxynucleotides (unlabeled) (Fig. 2). When an almost equivalent molar amount of poly(I)·poly(dC) was added to the incubation mixture before addition of enzyme, the rate of incorporation of [^{3}H]dTMP was markedly reduced. The poly(I)·poly(dC) has many more 3'OH termini per mole of nucleotide than 34S RNA·oligo(dT)$_{14}$. If the poly(I)·poly(dC) was added 1 min after the start of the reaction, no change in the rate of incorporation of [^{3}H]dTMP occurred. Thus, the $\alpha\beta$ form of AMV polymerase is processive in action, as is its associated RNase H activity.

Nonprocessive mode of action of polymerase subunit α. In contrast to the mechanism of action of the $\alpha\beta$ form of AMV polymerase, the α subunit appears to act in a nonprocessive fashion with synthetic primer-templates

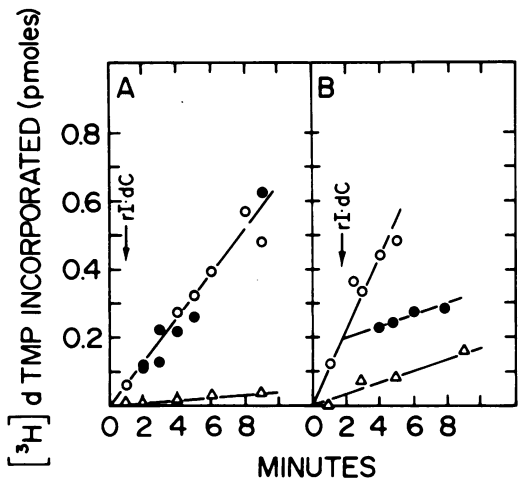

FIG. 2. *Mode of action of AMV polymerase and subunit α on 34S AMV RNA·oligo(dT)$_{14}$. AMV polymerase ($\alpha\beta$, 0.27 unit) or polymerase subunit α (0.2 unit) was incubated with 34S AMV RNA (0.602 nmol)·oligo(dT)$_{14}$ (0.136 nmol) with [^{3}H]dTTP (2,282 cpm/pmol, 35 nmol) and the other three unlabeled deoxynucleoside triphosphates as described in the text, except that the $MgCl_2$ concentration was 8 mM and actinomycin D was omitted. Poly(I) (0.36 nmol)·poly(dC) (0.11 nmol) was added to the incubation where indicated. (A) DNA synthesis with AMV polymerase ($\alpha\beta$); (B) DNA synthesis with AMV polymerase subunit α. Symbols: incorporation of [^{3}H]dTMP primed with 34S AMV RNA·oligo(dT)$_{14}$ alone, ○; after addition of poly(I)·poly(dC) at 1 or 1.8 min, respectively, after start of reaction, ●; addition of poly(I)·poly(dC) at start of reaction, △.*

poly(A)·poly(dT) and poly(I)·poly(dC). The initial rate of poly(dC) synthesis immediately decreased to the rate of synthesis observed with a mixture of the polymers (Fig. 3B) upon the addition of poly(A)·poly(dT) (Fig. 3C). The rate of poly(dT) synthesis was linear from the time of addition of poly(A)·poly(dT), in contrast to the delayed synthesis observed with the $\alpha\beta$ form of polymerase. The rate of poly(dT) synthesis, however, was not equal to the rate observed with the mixture of polymers. Reversal of the order of addition of polymers yielded the same results (Fig. 3D).

Polymerase subunit α also appears to act nonprocessively with 34S AMV RNA·oligo(dT)$_{14}$ (Fig. 2B). The rate of [^{3}H]dTMP incorporation decreased to the rate observed with the mixture upon addition of poly(I)·poly(dC), indicating that the polymerase subunit α may be equilibrating with all the polymers present in the incubation mixture. Grandgenett and Green (9) have shown that the AMV RNase H of the α subunit also appears to act nonprocessively.

The differences observed in the rates of DNA synthesis catalyzed by the two forms of polymerase in the polymer challenge experiments described above could reflect differences in their rates of association and/or dissociation with a polymer, rather than differences in their mode of action. To try to distinguish between these two possibilities, we incubated polymerase subunit α (0.72 unit) and polymerase $\alpha\beta$ (0.73 unit) with 34S AMV RNA (3.1 nmol) under conditions described in the legend to Fig. 1 and then measured the RNase H activity associated with each enzyme after addition of [^{3}H]poly(A)·poly(dT) as a measure of release of the enzymes. Similar experiments were carried out using ϕX DNA (1.6 nmol). These enzyme preparations contained 0.013 unit and 0.0075 unit of RNase H for subunit α and polymerase $\alpha\beta$, respectively (measured at 30°C as described below). RNase H activity was measured by the acid solubilization of [^{3}H]poly(A) (337 cpm/pmol, 15 pmol) annealed to poly(dT) (240 pmol) after 2, 5, and 10 min of incubation at 30°C. We found that the rates of RNase H activity observed with each enzyme were the same whether the [^{3}H]poly(A)·poly(dT) was added with the 34S AMV RNA or ϕX DNA or 1 min after the start of the incubation (data not shown). If one form of enzyme were more tightly sequestered than the other, we would expect differences in the rates of RNase H activity. The amount of RNase H activity detected with both enzymes was one-half that observed in the absence of ϕX DNA or 34S AMV RNA. These results suggest that the differences be-

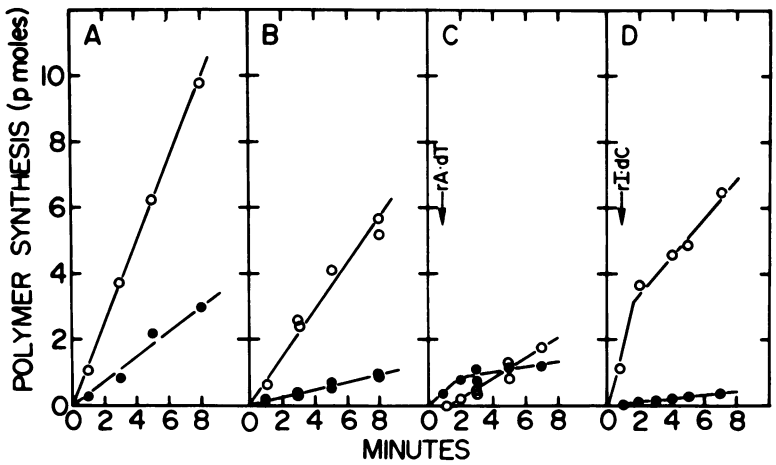

FIG. 3. *Nonprocessive mode of action of AMV polymerase on synthetic homopolymers. AMV polymerase subunit α (0.06 unit) was incubated with poly(A)·poly(dT) and/or poly(I)·poly(dC) as described in Fig. 1. (A) Each polymer pair was incubated with polymerase separately; (B) both polymer pairs were incubated with polymerase together; (C) enzyme was incubated with poly(I)·poly(dC) and poly(A)·poly(dT) was added 1 min after start of reaction; (D) enzyme was incubated with poly(A)·poly(dT) and poly(I)·poly(dC) was added 1 min after start of reaction. Symbols: poly(dT) synthesis, ○; poly(dC) synthesis, ●.*

tween the two forms of polymerase in the polymer challenge experiments reflect mode of action rather than simply rates of binding and release from polynucleotides.

Length of DNA transcripts formed with AMV polymerase αβ and 34S AMV RNA·oligo(dT). Since AMV polymerase (αβ) acts processively, it might be capable of completely transcribing 34S RNA in vitro. We therefore determined the size of the DNA transcripts formed with 34S AMV RNA·oligo$(dT)_{14}$ under conditions where AMV polymerase (0.2 unit) was incubated with 34S AMV RNA (306 pmol)·oligo$(dT)_{14}$ (177 pmol) for 2 h at 30°C. All of the DNA products were smaller than 5×10^5 daltons as analyzed by alkaline sucrose gradient centrifugation (Fig. 4A), as previously reported (17). When the amount of polymerase was increased to 3.3 units, 30% of the DNA product was found to have a sedimentation coefficient of 13S, corresponding to a molecular weight of 1.05×10^6 (Fig. 4A). More than 80% of the DNA formed with purified polymerase (4.8 units) was found to be of 1.05×10^6 molecular weight (Fig. 4B). The overall yield of DNA synthesized under these conditions was 40% relative to the amount of RNA template added. Thus, as the ratio of polymerase to 34S RNA was increased, larger DNA transcripts were formed. Significant amounts of larger DNA products have not been observed under the conditions tested. Addition of actinomycin D (60 μg/ml) did not affect the amount of 13S DNA synthesized.

It has been reported (4, 22) that for detergent-activated DNA synthesis in intact virions, the final concentration of deoxynucleotides influenced the size of the DNA transcripts made. We found that increasing the deoxynucleotide concentrations from those described in Fig. 4A to as high as 2.5 mM did not influence the size of the DNA transcripts formed in the in vitro reaction. Also, the sizes of the DNA products formed at high enzyme-to-template ratios with 34S AMV RNA·oligo$(dT)_{14}$, 34S RNA·$tRNA_{trp}$, or φX DNA·RNA hybrids (1% RNA sequences relative to DNA; formed with *Escherichia coli* RNA polymerase) were the same at all concentrations of deoxynucleotides tested (0.1 to 2.5 mM). Changes in the deoxynucleotide concentrations did increase the initial rates of DNA synthesis by as much as threefold, as well as the final yield of DNA synthesized in the reaction (less than twofold).

The DNA products formed with 2 or 4 units of polymerase subunit α were found to be four to five times smaller than the DNA products formed with the αβ polymerase. However, since we have detected small amounts of RNase in our α preparations (see above), the significance of this result is not clear.

The DNA product formed under conditions described in Fig. 4 with either form of polymerase was mostly single stranded (80%), as determined by its susceptibility to digestion by nucleases S1 and DNA exonuclease I. DNA samples were heated at 100°C for 5 min and then placed in ice before treatment with nuclease S1.

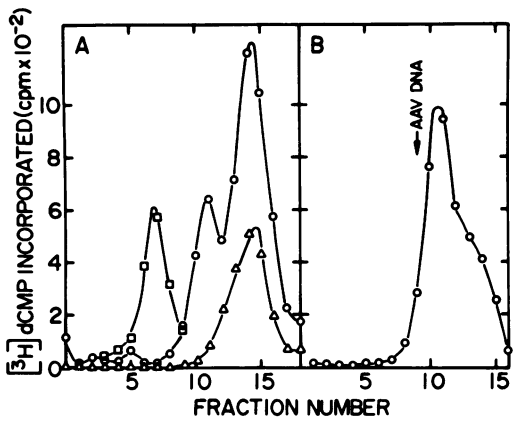

FIG. 4. *Alkaline sucrose gradient centrifugation of 34S AMV RNA·oligo(dT)$_{14}$-primed DNA transcript products. (A) AMV polymerase (0.2 unit or 3.1 units, αβ) was incubated with 34S AMV RNA (306 pmol)·oligo(dT)$_{14}$ (177 pmol) under conditions described in the text, except that the incubation mixture contained KCl (40 mM), ATP (0.4 mM), dATP, dGTP, and dTTP (0.5 mM each), and [^{3}H]dCTP (1,621 cpm/pmol, 0.063 mM). After 2 h of incubation at 30°C, 8,186 and 39,309 cpm of [^{3}H]dCMP was incorporated in the mixture containing polymerase 0.2 unit and 3.3 unit, respectively. DNA samples were analyzed on alkaline sucrose gradients. Centrifugation was at 49,000 rpm for 210 min as described in the text. ^{14}C-labeled φX DNA was included in the gradient as an internal standard. Symbols: △, DNA synthesis with polymerase (0.2 unit); ○, DNA synthesis with polymerase (3.3 unit); □, ^{14}C-labeled φX DNA. (B) AMV polymerase (4.8 units) and fraction P (3.1 units of polymerase) were incubated with 34S AMV RNA·oligo(dT)$_{14}$ as described in (A), except that actinomycin D (60 μg/ml, final concentration) was included in the incubation and α-[^{32}P]dCTP (1,895 cpm/pmol, 0.1 mM) was substituted for [^{3}H]dCTP. After 80 min of incubation, 44,163 cpm of [^{32}P]dCMP was incorporated into acid-insoluble form. The size of newly synthesized DNA was analyzed by alkaline sucrose gradients (centrifugation was for 180 min at 49,000 rpm) as described in the text. ^{3}H-labeled adeno-associated virus DNA (1.4 × 10^6) was included as an internal standard and its position is denoted by the arrow. Symbols: ○, DNA transcript.*

DISCUSSION

The results presented in this paper demonstrate that the αβ form of AMV polymerase, which represents the major form of polymerase isolated from virus, acts processively in catalyzing DNA synthesis. This means that once it binds to a primer-template the polymerase incorporates many deoxynucleotides before it dissociates. In contrast, the α subunit of polymerase appears to act nonprocessively. Analogous results have been reported for the α and αβ polymerase-associated RNase H activities (9, 15).

It should be noted that the experiments presented above measure changes in initial rates of DNA synthesis on synthetic and natural templates after challenge with a second template. The polymerase-catalyzed reaction consists of three steps: association, polymerization, and dissociation. If the α subunit has a much lower affinity than the αβ form of polymerase for the primer-templates tested, it would appear to act nonprocessively by this analysis. However, we have measured the K_m of binding to the synthetic polymers for both forms of polymerase under conditions described in Fig. 1 and have found that they are essentially the same. Subunit α has K_m of binding of 10^{-5} and 1.11×10^{-5} M for poly(I)·poly(dC) and poly(A)·poly(dT), respectively, whereas polymerase αβ has a K_m of binding of 1.2×10^{-5} and 0.9×10^{-5} M, respectively, for the same polymers. In addition, both forms of enzyme appear to be released from 34S AMV RNA at similar rates, as measured by RNase H activity with [^{3}H]poly(A)·poly(dT). These results suggest that the differences in rates of synthesis catalyzed by the two forms of polymerase in the polymer challenge experiments reflect mode of action. The possibility cannot be excluded that under conditions other than those tested, such as different temperature or pH, the α subunit may act processively.

The α subunit appears to be derived from the β subunit since all of the in vitro iodine-labeled, trypsin-resistant polypeptides prepared from the α subunit match peptides prepared from the larger α subunit (7). Also, Moelling (19) has presented data consistent with α subunits being derived from β subunits via proteolytic cleavage. Although catalytically active β subunits have not been purified from AMV, Hizi and Joklik have successfully purified an active ββ dimer from B77 (personal communication). In collaboration with Hizi and Joklik, we have shown that the ββ polymerase acts processively in transcribing poly(A)·oligo(dT)$_{14}$, poly(I)·oligo(dC)$_{12-18}$ poly(A)·poly(dT), and poly(I)·poly(dC). The αβ form of B77 polymerase also acts processively whereas the α form of polymerase appears to act nonprocessively, in agreement with the above results.

It is interesting to note that mammalian virus-derived reverse transcriptases resemble avian α subunits (9, 10, 22). The Friend leukemia virus polymerase (72,000 molecular weight) purified in this laboratory catalyzes DNA synthesis primed with 60S RNA and has

detectable RNase H activity. We find that both activities are nonprocessive in their mode of action (Goscin and Leis, unpublished data). Moelling (20) has recently reported the purification of a larger form of Friend leukemia virus polymerase of 84,000 daltons which has a processive RNase H activity. This enzyme may be comparable to the $\beta\beta$ form of the B77 enzyme.

The size of the DNA transcripts produced by the AMV polymerase was studied with 34S AMV RNA·oligo(dT)$_{14}$ instead of 60S RNA. The oligo(dT)$_{14}$ anneals solely to the 3′ terminus of the 34S AMV RNA so that the entire length of the 34S RNA is available for transcription. The tRNA$_{trp}$, which is responsible for much of the initiation of DNA synthesis on 60S RNA in vitro, has been shown to occupy a site near the 5′ terminus RNA (27; Leis and Smith, unpublished data). The largest DNA transcripts made with the processive $\alpha\beta$ form of AMV polymerase primed with oligo(dT)$_{14}$ were 1.05×10^6 daltons (or only about one-third the size of the RNA genome). Full-length DNA transcripts were not observed. The accumulation of 1.05×10^6-dalton transcripts may reflect the inability of the $\alpha\beta$ polymerase to transcribe through a region of strong secondary structure in the template. Once the enzyme reaches such a region, it may become sequestered or it may dissociate from the template. The kinetic experiments described in the text cannot distinguish between these two possibilities. The in vitro transcription of full-length complementary DNA by purified polymerase may require the addition of "RNA-binding" protein similar to the DNA-binding proteins known for bacterial systems (2, 11, 23). It is possible that such a protein is present in the virion core in limiting amounts, since there have now been several reports indicating that detergent-activated virus will produce small amounts of DNA 4,500 nucleotides or greater in length (4, 12, 22).

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