Human Immunodeficiency Virus 1 Reverse Transcriptase

TEMPLATE BINDING, PROCESSIVITY, STRAND DISPLACEMENT SYNTHESIS, AND TEMPLATE SWITCHING*

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The abbreviations used are: HIV-1, human immunodeficiency virus 1; AMV, avian myeloblastosis virus; kb, kilobase.

We have analyzed the kinetics of DNA synthesis catalyzed by reverse transcriptase from human immunodeficiency virus 1 (HIV-1). Reverse transcriptase, overproduced in *Escherichia coli* and purified to homogeneity, has polymerase and RNase H activity. Reverse transcriptase forms a stable complex with poly(rA)-oligo(dT) primer-templates in the absence of Mg**2+** and dTTP with an equilibrium dissociation constant of 3 nm. Synthesis from these preformed complexes can be initiated, and restricted to a single processive cycle, by the simultaneous addition of Mg**2+**, dTTP, and excess competitor RNA. Preformed complexes decay with a maximal half-life of 2–3 min. Synthesis on poly(rA) templates is processive with an incorporation rate of 10–15 nucleotides/s at 37 °C. Processivity varies widely with the template used, increasing from a few to >300 nucleotides in the order: poly(dA) < double-stranded DNA < single-stranded DNA < single-stranded RNA < poly(rA). On double-stranded DNA reverse transcriptase catalyzes limited strand-displacement synthesis of up to 50 nucleotides. On RNA-DNA hybrids significant DNA synthesis is observed only after degradation of the RNA strand by the RNase H activity of reverse transcriptase. Inter-molecular strand switching occurs with poly(rA) templates. At low ionic strength reverse transcriptase can use multiple templates with a single primer, leading to products of greater than template length. Reverse transcriptase and primer do not have to dissociate during the exchange of template strands, thus allowing processive DNA synthesis across template borders.

The human immunodeficiency virus 1 (HIV-1)† is the causative agent of the acquired immunodeficiency syndrome (AIDS) (see Fauci, 1988). A major target for treatment of AIDS infection is the viral reverse transcriptase (Mitsuya and Broder, 1987). Many inhibitors of reverse transcriptase are known; however, few are highly specific for the viral enzyme. The rational design of more specific inhibitors has been hampered by our lack of detailed knowledge on the molecular structure of reverse transcriptase and the mechanisms of its associated RNA-dependent DNA polymerase and RNase H activities.

Most of our knowledge about the function of reverse transcriptases comes from studies with the enzyme isolated from the avian myeloblastosis virus (AMV). Among the properties investigated are primer and template specificities (Balitmore and Smoler, 1971), kinetics and processivity of DNA synthesis (Fujinaga et al., 1970; Watson et al., 1973; Gregerson et al., 1980), interaction with tRNA (Penet et al., 1975; Haseltine et al., 1977), DNA unwinding and DNA binding activities (Collett et al., 1978; Matson and Bambara, 1981), fidelity of DNA synthesis (Battula and Loeb, 1976; Kunkel et al., 1981; Skinner and Eperon, 1986), function and mechanisms of its associated RNase H activity (Leis et al., 1973; Grandgenett and Green, 1974; Gerard, 1981; Champoux et al., 1984; Resnick et al., 1986), as well as the relationship between polymerase and RNase H activities (Brewer and Wells, 1974). Identification of amino acids located at or near the active site has been reported recently for the enzyme from the Moloney murine leukemia virus (Basu et al., 1988; Nanduri et al., 1988).

The reverse transcriptases from HIV-1, AMV, and Moloney murine leukemia virus all have polymerase and RNase H activities, but differ significantly in their structures (see Varmus and Swanstrom, 1984; Rho et al., 1981; Hansen et al., 1987). The HIV enzyme isolated from virus or virus-infected cells consists of two polypeptides, p66 and p51 (diMarzo Veronese et al., 1986; Lightfoot et al., 1986). The smaller one, being the result of carboxyl-terminal processing of p66 at an as yet unidentified site, lacks the presumptive RNase H domain and may also be deficient in polymerase activity (Starnes et al., 1988; Hansen et al., 1988). The significance of the processed polypeptide and the composition of the active enzyme are not clear at present. The Moloney murine leukemia virus enzyme is a monomer of 80 kDa with considerable sequence homology with the polymerase from HIV-1 (Johnson et al., 1986). The AMV enzyme is an α/β heterodimer consisting of a 63- and a 95-kDa subunit, each of which has polymerase and RNase H activity (see Varmus and Swanstrom, 1984). The β subunit also contains an endonuclease domain, a domain missing from the other two reverse transcriptases.

Since the first characterization of HIV-1 reverse transcriptase isolated from a human T-cell lymphoma cell line (Rho et al., 1981) basic properties of the purified enzyme such as optimal reaction conditions, and template and substrate specificities have been characterized extensively. Poly(rA) templates were generally found to give maximal incorporation rates with Mg**2+** as the preferred divalent cation. K**m** values for substrates of 10 μM, optimal salt concentrations of 50–100 mM, and optimal pH values around 8 have been reported (Rey et al., 1984; Hoffman et al., 1985; Wondrak et al., 1986; Cheng

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et al., 1987; Hansen et al., 1987). Most of the recent efforts have focused on expressing the HIV-1 enzyme in heterologous systems (Tanese et al., 1986; Farmerie et al., 1987; Hansen et al., 1987; Hizi et al., 1988) and on the search for specific inhibitors (Mitsuya et al., 1985; Furman et al., 1988; Cheng et al., 1987; Sobol et al., 1988). Sequence comparison (Johnson et al., 1986) and site-specific mutagenesis (Larder et al., 1987) have been used to roughly define the polymerase domain of HIV-1 reverse transcriptase. Little is known, however, about the kinetics of DNA synthesis. As part of an effort to understand the enzymatic mechanisms of HIV-1 reverse transcriptase, we have analyzed kinetic parameters of DNA synthesis using an enzyme overproduced in, and purified from, Escherichia coli.

**EXPERIMENTAL PROCEDURES**

**Materials**

Proteins—Plasmid pN6* (Adachi et al., 1986) harboring the HIV pol gene was obtained from M. Martin (National Institutes of Health, Bethesda, MD). The region encoding the reverse transcriptase was subcloned in an E. coli expression vector to yield an enzyme consisting of 560 amino acids. The amino terminus is identical to that of the enzyme purified from virus-infected cells and the carboxyl terminus has the sequence: Gly-Ile-Lys-Val-COOH (Gly being amino acid number 722 of the pol open reading frame; Ratner et al., 1985). Gel electrophoresis of the purified enzyme in the presence of sodium dodecyl sulfate shows two major bands corresponding to polypeptides with molecular masses of 66 and 51 kDa. The two polypeptides are present in an approximately equimolar ratio and account for >95% of the total protein. The 51-kDa polypeptide is the result of carboxyterminal processing by an E. coli protease. The concentration of active molecules in our preparation was determined by titration with template as described previously (Huber et al., 1987).

AMV reverse transcriptase was purchased from Pharmacia LKB Biotechnology Inc. Native bacteriophage T7 DNA polymerase, modified T7 DNA polymerase (a chemically modified form of the enzyme with an approximately 1000-fold reduced level of exonuclease activity), and T7 RNA polymerase were from the preparations described in Tabor et al. (1987) and Tabor and Richardson (1985, 1987), respectively. Proteinase K and DNAse I were purchased from Boehringer Mannheim.

Nucleotides—Nucleoside triphosphates were obtained from Pharmacia LKB Biotechnology Inc. Radioactively labeled nucleotides were from DuPont-New England Nuclear. The 7.5-kb poly(A)-tailed mRNA was from Bethesda Research Laboratory. All other polynucleotides were purchased from Pharmacia.

Plasmid mST109, constructed by S. Tabor (Harvard Medical School), is a derivative of M13mp7 containing the phage T7 610 promoter. A 1.2-kb DNA fragment from M13mp7 has been described in Tabor et al. (1987). Plasmid mST109, constructed by S. Tabor (Harvard Medical School), is a derivative of M13mp7 containing the phage T7 610 promoter. A 1.2-kb DNA fragment from M13mp7 has been described in Tabor et al. (1987). Plasmid mST109, constructed by S. Tabor (Harvard Medical School), is a derivative of M13mp7 containing the phage T7 610 promoter. A 1.2-kb DNA fragment from M13mp7 has been described in Tabor et al. (1987).

**Methods**

**Polymerase Assay for Reverse Transcriptase**—The following standard assay was used with appropriate modifications as indicated in the text. Reverse transcriptase at a concentration of 0.005–20 nM was preincubated with the appropriate primer-template for 5 min in 45 μl of 50 mM Tris-HCl, pH 8.0, 75 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Triton X-100 (buffer A). Desired enzyme dilutions were made in buffer A at 0 °C. Reactions were started by the addition of MgCl₂ and dNTP in 5 μl of buffer A to give final concentrations of 6 mM MgCl₂ and 150 μM dNTP. The nucleotide concentration was shown to be saturating. Reactions were stopped after the indicated time with 5 μl of 0.5 M EDTA. All incubations were carried out at 37 °C.

**Challenged Polymerase Assay**—In the challenged polymerase assay synthesis from preformed complexes of polymerase and primer-template is limited to a single processive cycle. This is achieved by addition of a polynucleotide, poly(U,G), at the reaction start that competes for binding of polymerase. The standard assay was modified as follows: enzyme and poly[A]₅₆₀-oligo(dT)₂₀ at the indicated concentrations were preincubated in a volume of 55 μl. Reactions were started by the addition of 15 μl of buffer A containing MgCl₂, 450 μM [³²P]dTTP (80 cpm/pmol), and 100 μg of poly(U,G) RNA. Reactions were stopped after 8 min. Even in the presence of competing RNA the enzyme incorporation inhibition occurs. The incorporation into poly(U,G) RNA could be monitored by adding [³²P]dUTP, and the kinetics of DNA synthesis was determined by site-specific primers (Larder et al., 1987).

**Other Polymerase Assays**—Experiments with native and modified bacteriophage T7 DNA polymerase were performed under conditions as described (Tabor et al., 1987). AMV reverse transcriptase was assayed under the same conditions as described above for the HIV-1 enzyme.

**Preparation of Primer-Template**—Primed single-stranded M13 DNA (mGP1-2) was prepared as described previously (Huber et al., 1987). Primed RNA was prepared from a discrete 7.5-kb mRNA and a 5'-³²P-labeled oligonucleotide (20-mer) complementary to a unique sequence close to the 3'-terminus of the RNA. Annealing was carried out at 65 °C in 5-fold molar excess of primer over RNA in 25 mM Tris-HCl, pH 7.5, 200 mM KCl, 0.2% sodium dodecyl sulfate, and 0.1 mg/ml proteinase K. Incubation was at 55 °C for 15 min, followed by slow cooling. Excess primer was removed by gel filtration over a CL-2B Sepharose column (Bio-Rad Laboratories).

**Double-stranded DNA Templates**—An oligonucleotide primer (17-mer) was annealed to the phage M13 DNA (mGP1-2) at a 5-fold molar excess as described (Huber et al., 1987). The primer was then extended by a 50-fold molar excess of T7 DNA polymerase for 30 at 37 °C after which the reaction was stopped with phenol/CHCl₃. Primer extension of greater than 2 kb was verified on an 0.8% agarose gel containing 0.5 μg/ml ethidium bromide. A second 5'-³²P-labeled oligonucleotide (19-mer, primer 1) was then annealed to the primer 2. Excess primers were removed by gel filtration through CL-2B Sepharose. The two primers are separated by a 4-base single-stranded gap. Randomly nicked pBR322 DNA was produced by incubating supercoiled plasmid DNA with DNase I (Millipore Corp.) until approximately 50% of the DNA was converted into replicative form II as judged by agarose gel electrophoresis.

**RNA-DNA Hybrid**—The RNA-DNA hybrid was obtained by hybridizing single-stranded M13 DNA and RNA synthesized by T7 RNA polymerase on the corresponding double-stranded DNA. A 1.2-kb mRNA was produced as a run-off transcript from double-stranded bacteriophage M13ST109 DNA linearized at the unique BstEII site. Reactions contained 50 mM Tris-HCl, pH 8.0, 20 mM MgCl₂, 5 mM dithiothreitol, 1 mM EDTA, 0.5 mM each of all four NTPs, 80 μg/ml linearized DNA, and T7 RNA polymerase (6000 units/ml). Reactions were stopped after 1 h at 37 °C by addition of sodium dodecyl sulfate (0.1%) and proteinase K (1 mg/ml, final concentration) and incubation for 20 min at 55 °C, followed by extraction with phenol/CHCl₃ and ethanol precipitation. Single-stranded mST109 DNA and a 2-fold excess of the 1.2-kb mRNA were annealed as described previously for primed RNA except that temperature was raised to 80 °C for 5 min before slow cooling. The RNA-DNA hybrid was purified by electrophoresis through a 0.7% low melting agarose gel. One aliquot of the hybrid was ³²P labeled at 3' end of the RNA strand by incubation with modified T7 DNA polymerase. Extensions were limited by the inclusion of only two dNTPs: 2 μM [α-³²P]dATP (3000 Ci/mmol) and 250 μM dGTP. Unincorporated nucleotides were removed by gel filtration through a Sephacryl G-200 column. The second 5'-³²P-labeled oligonucleotide (20-mer, primer 3) at 55 °C as described above. Excess primer was removed on CL-2B Sepharose. Primer 3 is complementary to the T7 610 promoter sequence immediately upstream of the 5'-end of the mRNA.

**Gel Electrophoresis**—Native and alkaline agarose gels and polyacrylamide gels in the presence of SDS were run as described (Maniatis et al., 1982; Tabor et al., 1987).

## RESULTS

The kinetics of DNA synthesis catalyzed by purified reverse transcriptase were analyzed using synthetic and natural single-stranded DNA and RNA templates primed with complementary oligodeoxynucleotides. Most kinetic parameters were determined with poly(rA) templates primed with

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1. J. M. McCoy and J. S. Sehra, manuscript in preparation.
2. J. M. McCoy and J. S. Sehra, unpublished results.
oligo(dT)$_{30}$ in a 1:1 molar ratio (expressed in 3' ends). The $K_a$ for the substrate, dTTP, was determined to be 10 ± 2 µM with this template (not shown). Under standard reaction conditions in the presence of template, reverse transcriptase is stable for more than an hour at concentrations as low as 10 pm.

**Preformed Complexes of Reverse Transcriptase and Primer-Template**

**Time Course of Incorporation**—The time course of DNA synthesis on poly(rA)$_{3000}$-oligo(dT)$_{30}$ primer-templates is shown in Fig. 1. When reverse transcriptase is incubated with poly(rA)-oligo(dT) in the absence of nucleotides a stable complex consisting of enzyme, primer, and template is formed. Synthesis from these complexes is initiated by the addition of dTTP and Mg$^{2+}$. Synthesis can be limited to a single processive cycle by trapping free and dissociating polymerase with competing oligonucleotides added at the reaction start. Several single- and double-stranded RNA and DNA polymers were tested for their effectiveness as competitors. Poly(U,G) RNA was found to trap reverse transcriptase more efficiently than either single-stranded DNA or RNA of random sequence. Furthermore, since poly(U,G) cannot serve as a template in the presence of dTTP alone it was used in all subsequent challenger experiments.

In the absence of competing RNA dTMP incorporation into poly(rA)$_{3000}$-oligo(dT)$_{30}$ is linear for more than 10 min. In a control reaction competitor RNA was included during the preincubation of polymerase and primer-template. In the presence of 2 mg/ml poly(U,G), synthesis is reduced to about 0.5%. With increasing primer-template concentration, this background incorporation will increase proportionally. If challenger RNA is added at the reaction start, incorporation takes off at a rate almost identical to that in the absence of challenger RNA. Within 5 min as all reverse transcriptase becomes trapped the rate of dTMP incorporation drops to background levels (Fig. 1). The plateau of incorporation is proportional to the initial concentration of preformed complexes of enzyme and primer-template. No assumptions about the nature of the reactions involved have to be made, i.e. about processivity of the polymerase reaction, possible proof-reading activities, or the dissociation kinetics of the preformed complex. Enzyme that is free at the time of challenger RNA addition becomes trapped immediately and does not participate in the subsequent reactions. This assay was used in the following experiments to determine kinetic parameters of the reverse transcriptase-template interaction.

**Binding Constant for the Interaction of Reverse Transcriptase and Poly(rA)-Oligo(dT)**—The affinity of reverse transcriptase for the synthetic primer-template was measured by titrating reverse transcriptase with increasing concentrations of poly(rA)$_{3000}$-oligo(dT)$_{30}$ (Fig. 2). Reverse transcriptase at three different concentrations and primer-template were allowed to equilibrate in the absence of nucleotides. The reactions were started by the addition of 150 µM [3H]dTTP and excess challenger RNA as described above. The final incorporation in this assay is again proportional to the concentration of the preformed complexes of enzyme and primer-template. The incorporation as a function of the primer-template concentration is shown in Fig. 2. The equilibrium dissociation constant, $K_d$, of the polymerase-primer-template interaction was determined as described in the legend to Fig. 2. Values of 2.3 ± 1 µM template and 3.4 ± 0.4 µM were obtained from the template concentration giving 50% maximal incorporation and from curve-fitting, respectively.

Reverse transcriptase binds specifically to the double-stranded section of the primer-template of poly(rA)-oligo(dT). Binding to the single-stranded part of the template

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**Fig. 1.** Time course of DNA synthesis by preformed complexes of reverse transcriptase and poly(rA)-oligo(dT). Reverse transcriptase (4 nM) and poly(rA)$_{3000}$-oligo(dT)$_{30}$ (20 nM) were preincubated as described under "Experimental Procedures." Reactions were started by the addition of Mg$^{2+}$, [3H]dTTP, and either 2 mg/ml challenger RNA (poly(U,G)) (■) or no challenger RNA (■). In a control reaction, poly(U,G) was included during the preincubation and the reaction was started with Mg$^{2+}$ and dTTP (▲). Reactions were stopped at the indicated time. Concentrations are those of the final reaction mixture.

**Fig. 2.** Titration of reverse transcriptase with primer-template. Reverse transcriptase at a concentration of 16 (●), 8 (□), or 4 nm (▲) was preincubated with poly(rA)$_{3000}$ poly(dT)$_{30}$ of the indicated concentrations. Reactions were started by the addition of Mg$^{2+}$, [3H]dTTP, and 2 mg/ml challenger RNA and stopped after 6 min. Concentrations given are those in the final 50-µl reaction. The equilibrium dissociation constant $K_d$ was determined in the following manner. The equilibrium during preincubation can be described by the scheme $E + S ⇌ ES$, where $E$ and $S$ are the free enzyme and primer-template, respectively. Incorporation after the reaction start with excess challenger RNA is proportional to the complex concentration $[ES]$ and maximal when $[ES] = [E]$, independent of the processivity or proofreading activity of the enzyme. Combining the equations

$$K_d = \frac{[E][S]}{[ES]}$$  \hspace{1cm} (1)

$$[E] = [E] + [ES]$$  \hspace{1cm} (2)

$$[S] = [S] + [ES]$$, and

$$[ES] = \frac{[E][S]}{K_d}$$ \hspace{1cm} (3)

at the template concentration giving 50% maximal incorporation, we obtain

$$K_d = [S] - \frac{[E]}{2}$$ \hspace{1cm} (4)

Alternatively, $K_d$ was determined by curve fitting using Equations 1-3. [ES] was calculated as a function of [S] and $K_d$, $K_d$ was then varied to give an optimal fit of the [ES] versus [S] curve with the experimental data.
Dissociation of Preformed Complexes—Complexes of reverse transcriptase and poly(rA)-oligo(dT) are in equilibrium with free polymerase and primer-template. Dissociation of the complexes can be monitored in the presence of competing poly(U,G) RNA (Fig. 3). Poly(U,G) traps dissociating reverse transcriptase, thereby preventing its reassociation with template. Preformed complexes were exposed to poly(U,G) for the indicated time before the polymerase reaction was started. At the standard salt concentration of 75 mM KCl, the complexes decay with a half-life of 120 ± 30 s. In the absence of KCl, the complexes are more stable with a half-life of 200 ± 50 s. Over most of the monitored time interval the kinetics of dissociation follow a first order reaction, independent of the concentration of competing RNA. A fraction of the complexes decays rapidly within the first minute, a phenomenon also observed with other polymerases (Huber et al., 1987).

Primer Extension Is Synchronous—The completion of a single cycle of processive synthesis requires several minutes (Fig. 1). This result may be due to a low incorporation rate on a long template of variable length or to delayed initiation of synthesis by a fraction of the preformed complexes. To distinguish these possibilities, primer extension by preformed complexes of enzyme and primer-template was followed with time.

Reverse transcriptase was preincubated with poly(rA)300 primed with 5' 32P-labeled oligo(dT)20. Reactions were started with Mg2+ and dTTP, stopped at the indicated time, and the products were separated on a urea-polyacrylamide gel (Fig. 4). Primer extension occurs essentially synchronously and allows us to estimate the incorporation rate. On poly(rA) dTTP incorporation occurs at an average rate of 10–15 nucleotides/s at 37 °C, a rate corresponding to the actual elongation rate since synthesis is processive on poly(rA) (see below).

Processivity of Reverse Transcriptase

Processivity of a polymerase is defined as the average number of nucleotides incorporated into a given template before enzyme and template dissociate. Replicative polymerases are in general processive, either by themselves or in association with accessory proteins, to allow efficient genome replication by a small number of polymerase molecules (see Kornberg, 1980). Since HIV reverse transcriptase is a replicative polymerase, we wanted to test whether the enzyme by itself was processive.

Processivity on Homopolymeric Templates—Processivity on single-stranded templates was measured by the extension of labeled primers. Two criterions for processivity were used. Under conditions of limiting polymerase each primer extension is due to a single association-dissociation event and thus is a direct measure of processivity. Furthermore, patterns of primer extension that do not change with increasing polymerase concentration are the result of processive synthesis.

Poly(rA) primed with 5' 32P-labeled oligo(dT)20 was extended with reverse transcriptase of increasing concentration in the presence of 75 mM KCl (Fig. 5, first 5 lanes). At the lowest reverse transcriptase concentration, only a tiny fraction of the available primers are being used. With increasing reverse transcriptase concentration, the amount of products rather than the product distribution changes. The extension of primers by several hundred nucleotides is therefore the results of processive synthesis. Processivity at lower ionic strength is even higher (not shown).

In contrast, processivity and rate of incorporation on the DNA template poly(dA) are very low (Fig. 5, lanes 6 to 10). Under conditions where the majority of the primers have been used (1.2 nM polymerase), most are extended by 1–20 nucleotides. Lowering the KCl concentration to zero increases the
incorporation rate on poly(dA) but not the processivity (not shown). Reverse transcriptase is less processive on poly(C) and poly(dC) templates primed with oligo(dG) than on poly(rA) (not shown). Products analyzed on alkaline agarose gels indicated that reverse transcriptase is processive on both templates but for less than 100 nucleotides.

Single-stranded DNA Templates of Random Sequence—M13 single-stranded DNA was used to estimate processivity on DNA templates of random base composition. A time course of extension of 32P-labeled primers at 75 mM KCl is shown in Fig. 6A. Although there is a set of distinct stops at low polymerase concentrations, essentially all primers can be extended to full-length products with excess enzyme. Processivity on single-stranded M13 DNA is considerably higher than on the homopolymer poly(dA) (Fig. 6B). A titration with increasing reverse transcriptase demonstrates that all extensions seen at the lowest concentration are the products of processive synthesis. The longest processive extensions are in the range of 200–300 bases with an average processivity of approximately 50 nucleotides.

The pattern of stops does not seem to be caused by template secondary structure. There are no stable hairpins in the analyzed region of the template and raising the temperature to 42 °C (Fig. 6B) or lowering the KCl concentration to 25 mM does not affect the pattern. Analysis of the primary sequence suggests that most strong stops occur within 1 or 2 bases after sequences containing at least three A and/or T residues in a row. This correlation is consistent with the finding that reverse transcriptase is nearly distributive on a poly(dA) template (Fig. 5).

Single-stranded RNA Templates—A mRNA of 7.5 kb length with a DNA 17-mer annealed at its 3’ end was used as RNA template. Primer extension on this template is qualitatively similar to that on single-stranded DNA. Maximal processivity is greater than 300 nucleotides with an average processivity of approximately 100 nucleotides (Fig. 7A). Varying the KCl concentration does affect the incorporation rate, but neither processivity nor the pattern of stop sites are significantly influenced by the ionic strength (B). Similarly, there is no dependence of processivity on pH within the tested range of 7.0–8.3 (not shown). Again, the pattern of stops seems unrelated to secondary structure, in that they are independent of the salt concentration.

The comparison of the reverse transcriptases from HIV-1 and AMV indicates that most of the stop sites are common to both enzymes, although their relative intensities vary widely. Examination of the stop sites in the sequence context revealed no obvious correlation.

Template Switching by Reverse Transcriptase
Salt Dependence of Product Length—In general, the length of a newly synthesized DNA, being a copy of the template strand, will not exceed that of the template. On a 460-base template randomly primed with a 20-mer, the average product length should therefore be 220 nucleotides with an upper limit of approximately 440 nucleotides. Surprisingly, on poly(rA)460-oligo(dT)5 many longer products can be synthesized by reverse transcriptase. The size distribution of products obtained at various salt concentrations was analyzed on alkaline agarose gels (Fig. 8A). Products formed in the presence of 150 mM KCl are predominantly less than 440 bases in length; the small population of larger products can be explained by the variation in length of the individual templates.
Reverse Transcriptase from HIV-1

At lower salt concentrations products of several thousand bases in length are observed. Products longer than the template may arise by three mechanisms: 1) primer slippage along homopolymeric templates to allow continuous use of the same template; 2) relocation of the primer terminus to a new template (intermolecular template switching); 3) relocation of the primer terminus to the 3' end of the template after the template has been copied up to the 5' end (intramolecular template switching). The latter mechanism can be distinguished from the first two in that it should be dependent on template concentration.

Dependence of Product Length on Template Concentration—The effect of template concentration is shown in Fig. 8B. Poly(rA)₆₀-oligo(dT)₂₀ (0.01 μM) and poly(rA)₆₀ of increasing concentration were incubated with reverse transcriptase and [α⁻³²P]dTTP in the absence of KCl. The product length clearly increases with increasing template concentration. This result indicates that relocation of the primer 3' end to new templates is, at least in part, responsible for the observed long products. Products obtained over a 100-fold concentration range of reverse transcriptase are qualitatively identical (Fig. 8B), i.e. the product length increases with template concentration at all concentrations of polymerase. Alternating template switching and primer extension of a given primer take place even under conditions of limiting polymerase. Reverse transcriptase must, therefore, be able to remain bound to the DNA strand while the RNA template is exchanged. As a result, processive synthesis beyond the limits of template length is possible.

DNA synthesis on double-stranded templates requires the displacement of the non-template strand downstream of the polymerization site. Although strand displacement synthesis occurs at replication forks with the help of helicases, some polymerases by themselves are capable of initiating and sustaining strand displacement synthesis at a nick within double-stranded DNA. Strand displacement synthesis can be followed by intramolecular strand switching. This type of strand switching involves branch migration and the relocation of the primer terminus from the original template strand to the displaced strand leading to branched structures (see Kornberg, 1980).

Double-stranded DNA Templates—Randomly nicked pBR-322 plasmid DNA was used to test whether reverse transcriptase can initiate strand displacement synthesis on a nicked double-stranded DNA template. The products obtained in the presence of α⁻³²P-labeled nucleotides were separated on agarose gels containing ethidium bromide (Fig. 9). One major product is labeled by reverse transcriptase and runs at a position corresponding to open circular DNA (lane 1). Lanes 2–4 show labeled plasmid standards obtained with T7 DNA polymerase. Lane 4 indicates the position of panhandle structures produced by modified T7 DNA polymerase. Panhandles are formed by strand displacement synthesis at a nick followed by strand switching. They exhibit unique mobility during electrophoresis in the presence of ethidium bromide because of topological constraint (Lechner et al., 1983). No panhandles are observed with reverse transcriptase, i.e. no strand switching occurs.

The labeling of nicked double-stranded templates by reverse transcriptase is the result of strand displacement synthesis and not of excision-incorporation cycles at the nick.

**FIG. 8. Variation of product length on poly(rA) templates.**

A, salt-dependence. Reactions with 2 nM reverse transcriptase and 8 nM poly(rA)₆₀-oligo(dT)₂₀ at the indicated KCl concentration were initiated as described under “Experimental Procedures” and stopped after approximately 80% of the 5' ³²P-labeled primers had been extended (5–15 min). Reaction products were separated on a 1.5% alkaline agarose gel. Molecular weight markers are shown in the first lane. B, template dependence. Reverse transcriptase of the indicated concentration was incubated with 10 nM poly(rA)₆₀-oligo(dT)₂₀ in the absence of KCl. Reactions were started by addition of Mg²⁺, [α⁻³²P]dTTP, and poly(rA)₆₀ of the indicated final concentration and stopped after 15 min. Products were separated on a 0.8% alkaline agarose gel.

**FIG. 7. Processivity on RNA.** Reactions with reverse transcriptase and 2 nM of a 7.5-kb mRNA primed with a 5' ³²P-labeled DNA 17-mer were initiated as described under “Experimental Procedures” and stopped after 10 min. A, dependence of primer-extension on enzyme concentration at 75 mM KCl. B, dependence on KCl concentration at a reverse transcriptase concentration of 0.1 nM. C, comparison of products synthesized by reverse transcriptases from HIV-1 and AMV at 75 mM KCl. Enzyme concentrations were 10 nM (HIV-1) and 40 units/ml (AMV), respectively.

**Strand Displacement Synthesis**
that occurs with polymerases that have exonuclease activities. This point is illustrated in Fig. 10. The template in this experiment was single-stranded M13 DNA to which two oligonucleotide primers were annealed. The two primers are separated by a 4-base single-stranded gap. Primer 1, labeled with $^{32}$P at the 5' end, is located upstream of primer 2. Where indicated, primer 2 was extended by several hundred nucleotides to form a long double-stranded stretch of DNA downstream of primer 1 (lanes 5 and 6). The extension of primer 1 by reverse transcriptase in the absence of primer 2 is shown at two salt concentrations (lanes 1 and 2). In the presence of primer 2, a strong stop occurs at position -1 (lanes 3 and 4), but most of the products are the same as in its absence, indicating primer displacement. At the higher ionic strength, more primer extensions end within the duplex region of primer 2 due to its increased stability (lane 4).

When the double-stranded DNA stretch is much longer, strand displacement comes to a halt within some 50 base pairs (lanes 5 and 6). The major stops are identical to those in the presence of unextended primer 2, confirming that strand switching does not occur to a significant degree. At the lower ionic strength the distribution of products is shifted slightly towards longer size. The observed primer extensions are the result of processive strand displacement synthesis, since the pattern of stops does not change with the concentration of reverse transcriptase (not shown). The absence of strand switching by reverse transcriptase is probably due to a more stringent requirement for homology between primer and template than is the case with T7 DNA polymerase. Alternatively, short panhandle structures may form but may not be stable in the presence of intercalating agents. Lanes 7 and 8 show controls for the presence and absence of strand displacement synthesis as observed with modified and native T7 DNA polymerase, respectively.

**RNA-DNA Hybrid Template**—To test for strand-displacement synthesis on an RNA-DNA hybrid, a 1.2-kb complementary mRNA was annealed to single-stranded M13 DNA (mST109). Immediately upstream of the mRNA a 5' $^{32}$P-labeled oligonucleotide (20-mer) was annealed. The extension of this primer by reverse transcriptase in the absence or presence of RNA is shown in Fig. 11, A and B. In the absence of a double-stranded region, the number of primers extended is approximately proportional to the enzyme concentration while the product length (i.e., processivity) is invariant. In the presence of the double-stranded region downstream, the number of primers used is no longer proportional to reverse transcriptase concentration, suggesting a more complex reaction than simple strand displacement. Analysis of the RNA strand demonstrates that appreciable primer extension only takes place after most of the RNA has been degraded by the RNase H activity (Fig. 11C). In this control experiment the $^{32}$P label was at the 3' end of the RNA strand of an otherwise identical template. Reverse transcriptase and template were incubated in the absence of nucleotides. The RNA is almost completely degraded at an enzyme concentration of 2 nM, with resulting 3' fragments ranging in size from 9 to 65 nucleotides. The same result was obtained in the presence of all four dNTPs and when using uniformly $[^{32}$P]UMP-labeled RNA instead of end-labeled RNA. Agarose gel electrophoresis of the products obtained at a reverse transcriptase concentration of 20 nM reveals that the RNA is not only degraded but
A primer-template consisting of single-stranded M13 DNA (mST109) to which a 5' 32P-labeled oligonucleotide (20-mer) had been annealed was used in the reactions shown in A. In B a 1.2-kb mRNA had been annealed to the primer-template immediately downstream of the primer. In C the same template was used as in B, except that the 3' end of the mRNA was 32P-labeled instead of the primer (see “Experimental Procedures”). Reactions with reverse transcriptase of the indicated concentration were performed under standard conditions except in C where the nucleotides were omitted. Reactions were stopped after 30 min and products were separated on 8% urea-polyacrylamide gels.

also completely displaced from the single-stranded DNA template (not shown). Strand displacement synthesis on an RNA-DNA hybrid thus appears not to contribute significantly to second strand synthesis in vitro.

**DISCUSSION**

The replication of the RNA genome of HIV-1 most likely proceeds through the same complex sequence of events identified with other retroviruses, including tRNA-primed continuous minus-strand synthesis, template switching, RNA degradation, and plus-strand synthesis that requires strand displacement and a second template switch. To test which of these steps can be catalyzed by HIV-1 reverse transcriptase and which may require additional factors, we have analyzed DNA synthesis with the purified enzyme on various single- and double-stranded templates.

Reverse transcriptase displays a strong affinity for poly(rA)-oligo(dT) primer-templates with a binding constant of approximately 3 nM. Similar values have been found with the processive DNA polymerases from phages T5 and T7 on poly(dA)-oligo(dT) templates (Das and Fujimura, 1980; Huber et al., 1987), whereas the nearly distributive E. coli DNA polymerase I has a 70-fold lower affinity (Bryant et al., 1983). Reverse transcriptase binds to the primer-template junction of poly(rA)-oligo(dT) in the absence of nucleotides and divalent cations to form an enzyme-primer-template complex. Synthesis from such preformed complexes can be initiated and restricted to a single processive cycle by the addition of Mg2+, dTTP, and excess competing RNA. Synthesis starts synchronously and proceeds at 10–15 nucleotides/s. This elongation rate, measured during processive synthesis, is in the same range as those of eukaryotic DNA polymerases. However, it is 10–20-fold higher than those reported for reverse transcriptases from other retroviruses (see Varmus and Swanstrom, 1984). This discrepancy may arise from DNA synthesis being less processive on RNA and DNA templates of random sequence (see below).

The preformed complexes of reverse transcriptase and primer-template dissociate with a half-life of several minutes, as do the analogous complexes formed with the processive bacteriophage DNA polymerases. The dissociation kinetics show that a fraction of the complexes is much less stable and decays within seconds rather than minutes. This apparent heterogeneity in dissociation properties may reflect two interconvertible forms of preformed complexes as has been postulated in the case of E. coli DNA polymerase I (Bambara et al., 1976). Alternatively, it may be due to a heterogeneity in the enzyme preparation. The presence of two polypeptides, p51 and p66, leaves open the possibility of formation of homodimers with potentially different properties.

Processivity of HIV-1 reverse transcriptase on poly(rA) templates is greater than 300 nucleotides and increases with lower ionic strength, similar to that of the enzyme from AMV (Gregerson et al., 1980). Processivity on other templates varies widely, being lowest on poly(dA). The number of oligo(dT) primers extended per min, however, is about the same on poly(rA) and poly(dA) templates and explains the much lower incorporation rates on the DNA template (Rho et al., 1981; Hoffman et al., 1985). Processivity on RNA and DNA templates of random base composition is similar, 50–100 nucleotides on average at 75 mM KCl, and does not increase significantly at lower ionic strength. This value appears very low for a replicative DNA polymerase. However, in vitro reverse transcriptase is likely to be part of a larger nucleoprotein complex. Strong evidence for tight and obligatory association of the polymerase with the viral particle or in complementation experiments with mutants of Rous sarcoma virus (Hanafusa et al., 1972). A mutant deficient in reverse transcriptase cannot be rescued by coinfection with a wild-type strain. The apparent processivity in such a nucleoprotein complex may be increased through simple physical restraint of the polymerase or by interaction with accessory proteins provided by either virus or host.

Synthesis on poly(rA) can lead to products of greater than template length. A similar phenomenon, involving slippage of primers along templates of repetitive sequence, has been observed with E. coli DNA polymerase I (see Kornberg, 1980). Although sliding of the oligo(dT) primer along the poly(rA) template may account in part for the observed effect with reverse transcriptase, we show that a second mechanism, intermolecular template switching, is involved as well. Switching of poly(rA) templates by reverse transcriptase may be facilitated by its intrinsic RNase H activity. Template switching can occur processively, i.e. reverse transcriptase remains with the primer while changing templates, thereby allowing processive synthesis across template borders. This ability is particularly intriguing in light of the requirement for reverse transcriptase to jump templates during replication of the linear retroviral genome. Synthesis of both minus- and plus-strand DNA requires a template switch involving repeated sequences near the ends of the genome (see Gilboa et al., 1979; Varmus, 1983).

HIV reverse transcriptase has no exonuclease activity. It can, therefore, be expected to catalyze strand displacement synthesis like other polymerases with low 3'–5' DNA exonu-

**FIG. 11. Synthesis on a double-stranded DNA-RNA hybrid.**
clease activity (Masamune and Richardson, 1971; Fujimura and Roop, 1976; Lechner et al., 1983). Limited strand displacement synthesis does occur on double-stranded DNA, a nick being sufficient for initiation. This finding contrasts with in vitro results with reverse transcriptases from other retrovirus (Hurwitz and Leis, 1972) but is consistent with observations of DNA strand displacement in vivo (Boone and Skalka, 1981; Hsu and Taylor, 1982). Strand displacement synthesis through a short stretch of double-stranded DNA is required by the favored model for second strand synthesis (see Varmus and Swanstrom, 1984, 1985). Template switching during strand displacement synthesis could not be detected. This type of intramolecular strand switching from the original template to the displaced strand would be deleterious for a replicative polymerase and is probable prevented by a stringent requirement of the reverse transcriptase for extended homology between primer and template. Under identical conditions, the modified T7 DNA polymerase switches templates readily during strand displacement synthesis (Lechner et al., 1983); however, the phage enzyme is known for its ability to use primers of minimal homology.

The combination of polymerase and RNase H activities on a single molecule suggests the interesting possibility that the two activities may be mechanistically coupled. Synthesis of the second cDNA strand and RNA degradation might occur concomitantly in a manner analogous to nick translation by E. coli DNA polymerase I (see Kornberg, 1980). However, this does not seem to be the case in vitro. A double-stranded RNA-DNA hybrid was used to mimic the situation during the synthesis of the retroviral plus-strand. Parallel experiments that monitor primer extension and RNA strand degradation indicate that synthesis of the second cDNA strand in vitro takes place only after most of the RNA has been degraded and displaced from the DNA. The nonproportionality between enzyme concentration and synthesis on the hybrid template further supports the conclusion that DNA synthesis and RNA degradation are not coupled events, but are catalyzed by distinct polymerase molecules in an sequential manner. Conversely, the creation of potential RNA primers by RNase H is not automatically followed by primer extension. Only a small subpopulation of the primers is used, and their extension occurs with a very low efficiency (results now shown).

This selectivity likely reflects the in vivo requirement to use primers of minimal homology.

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Note Added in Proof—A detailed kinetic analysis of HIV-1 reverse transcriptase isolated from viral particles has been reported recently (Majumdar, C., Abbott, J., Broder, S., and Wilson, S. H. (1988) J. Biol. Chem. 263, 15657–15665).

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