Reverse Transcriptase and Its Associated Ribonuclease H: Interplay of Two Enzyme Activities Controls the Yield of Single-Stranded Complementary Deoxyribonucleic Acid†

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ABSTRACT: The synthesis of single-stranded globin cDNA by the RNA-directed DNA polymerase activity of reverse transcriptase in the presence of oligothymidylate primers was investigated in order to determine the limitations to higher yields. The results indicated that the associated ribonuclease H activity, an integral part of reverse transcriptase, plays a large role in the synthesis of the *first strand* of cDNA and that the interplay of the two enzyme activities for any specific set of conditions determines the yield of single-stranded products. In both the presence and the absence of polymerization, the associated ribonuclease H catalyzed the deadenylation of mRNA, producing molecules that were somewhat shorter, highly homogeneous in size, and fully translatable into globin protein. They were also entirely lacking in the ability to serve as templates for cDNA synthesis. The reaction was completely dependent on oligothymidylate and completely independent of deoxyribonucleoside triphosphates. The initial rate of deadenylation was one-fourth the initial rate of initiation of polymerization when saturating levels of deoxyribonucleoside triphosphates were used in the polymerase reaction. In the presence of ribonuclease H activity, the DNA polymerase catalyzed the synthesis of an array of cDNAs including some that were full length. The initiation of polymerization was rate limiting: once synthesis had begun, it required 1–1.5 min to transcribe globin mRNA. However, most primers that were elongated were aborted prematurely. Maximum synthesis of full-length cDNA required stoichiometric levels of enzyme and high triphosphate levels, but regardless of conditions, the sum of completed cDNA and deadenylated mRNA accounted for only 50% of the input mRNA. The data fit a model in which synthesis of full-length cDNA molecules depends on the arrangement of primers and transcription initiation complexes on the poly(A) "tail" of mRNA.

Reverse transcriptase from avian myeloblastosis virus is a key ingredient in recombinant DNA technology (Taylor et al., 1977; Papas et al., 1981; Efstratiadis & Villa-Kamaroff, 1979). Since the enzyme is essential for the synthesis of single-stranded complementary DNA, the initial step in cloning, it is clearly important to optimize yields. Losses sustained during cDNA synthesis place subsequent steps of cloning at a disadvantage, particularly if the desired nucleic acid sequence is rare. Although many studies have aimed at improving the quality and the quantity of the first cDNA strand (Kacian & Myers, 1976; Monahan et al., 1976; Buell et al., 1978; Retzel et al., 1980), attempts at understanding the need for a particular set of conditions (Payvar & Schimke, 1979) have generally not been undertaken.

From the enzymological point of view, the protocols that have evolved are bizarre. The concentrations of primer, deoxyribonucleoside triphosphates, and reverse transcriptase are apparently excessive. For example, the concentration of reverse transcriptase advocated by several investigators is stoichiometric, rather than catalytic (Papas et al., 1981; Wickens et al., 1978; Okayama & Berg, 1982). If it is assumed that the $\alpha\beta$ form of the protein has two active sites (Kacian et al., 1971; Grandgenett et al., 1973; Verma, 1975; Grandgenett, 1976; Hizi & Joklik, 1977), the use of 10–50 units of enzyme activity per μg of mRNA, a standard figure, results in equimolar quantities of reverse transcriptase and template for small mRNAs such as globin. For mRNAs greater than 28 S in size, the molar enzyme concentration exceeds the molar RNA concentration by 20-fold. Similarly, deoxyribonucleoside triphosphates (dNTPs)[1] used in the millimolar concentration range (Wickens et al., 1978; Okayama & Berg, 1982; Puskas et al., 1982) are commonplace despite the fact that these materials apparently become rate limiting below a much lower value, 75 μM (Retzel et al., 1980; Collett & Faras, 1977). In the present study, part of an ongoing project to clone nonabundant mRNAs (Wallace et al., 1981; Gray et al., 1982), we have set out to determine why the yield of full-length cDNA rarely exceeds 40%, regardless of conditions. In doing so, we find that the interplay of two catalytic functions determines the yield of product.

Reverse transcriptase consists of two activities that are both an integral part of the enzyme: a DNA polymerase activity and a ribonuclease H (RNase H) activity (Molling et al., 1971; Keller & Crouch, 1972; Baltimore & Smoler, 1972; Leis et al., 1973). The associated RNase H is an exonuclease capable of cleaving the RNA strand of a DNA–RNA hybrid into 5′-phosphoryl oligomers roughly 2–30 nucleotides long (Leis et al., 1973; Verma, 1981). The reaction has been studied extensively with poly(A)·poly(dT) as the substrate; with poly(A)·oligo(dT), little or no cleavage of the RNA strand was observed (Leis et al., 1973; Grandgenett & Green, 1974) unless TTP for polymerization was also provided. It was believed that RNase H activity lay dormant until elongation of the primer by the polymerase activity generated a suitable substrate (Watson et al., 1979). Then, by degrading the mRNA, the ribonuclease H activity cleared the way for synthesis of the anticomplementary (second) DNA strand (Molling et al., 1971).

Here we show that the prevailing view of the role played by the associated ribonuclease H is incomplete and partly

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[1] Abbreviations: RNase H, ribonuclease H; Cl_3CCOOH, trichloroacetic acid; ACE, 10 mM sodium acetate, 3 mM EDTA, and 50 mM NaCl at pH 5.1; dNTPs, deoxyribonucleoside triphosphates; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

incorrect. In the presence or absence of deoxyribonucleoside triphosphates for polymerization, the associated RNase H is able to deadenylate globin mRNA by using the hybrid formed between the poly(A) tail and oligo(dT) as a substrate. Removal of the tail abolishes synthesis of the first strand of cDNA owing to the loss of affinity of the mRNA for the oligo(dT) primer. Both the polymerase and the ribonuclease H are active immediately upon mixing the components necessary for the synthesis of cDNA. The effect of the concentration of enzyme and substrates on the two competing reactions during synthesis of the first strand of cDNA has been evaluated, and a model which is consistent with the need for both stoichiometric levels of enzyme and extraordinarily high triphosphate concentrations has been devised.

Materials and Methods

Materials. Globin messenger RNA was obtained from BRL (Gaithersburg, MD) and used without further purification. The poly(A) tails, on the average, were 40 residues long as shown by [^{3}H]poly(U) hybridization (Safer et al., 1979). Oligo(dT)$_{12-18}$ and 5′-dephospho-oligo(dT)$_{12-18}$ were purchased from P-L Biochemicals (Milwaukee, WI). Enzymes and their suppliers were as follows: reverse transcriptase (7.3 units/pmol), Life Sciences, Inc. (Gulfport, FL); polynucleotide kinase, P-L Biochemicals; poly(A) polymerase, BRL. Wheat germ for translating mRNAs was part of a kit from BRL. Radioactive compounds and their sources included L-[^{35}S]-methionine (>1000 Ci/mmol), either Amersham (Arlington Heights, IL) or New England Nuclear (Boston, MA), adenosine 5′-[γ-^{32}P]triphosphate (3000 Ci/mmol), adenosine 5′-[α-^{32}P]triphosphate (~2000–3000 Ci/mmol), and deoxyguanosine 5′-[α-^{32}P]triphosphate, deoxycytidine 5′-[α-^{32}P]-triphosphate, and deoxyadenosine 5′-[α-^{32}P]triphosphate (each at 635 Ci/mmol, Amersham).

Reverse Transcription of mRNA. The preparation of cDNA was carried out at 37 °C as described previously (Puskas et al., 1982). Briefly, reaction mixtures contained 50 mM Tris-HCl at pH 8.3, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA. Unless noted otherwise, the concentrations of globin mRNA and oligo(dT) were 20 and 5 μg/mL, respectively. The concentrations of the four deoxyribonucleoside triphosphates and reverse transcriptase varied and are specified in the individual figure legends and tables. A stock solution of reverse transcriptase at 11 700 units/mL in 0.2 M potassium phosphate at pH 7.2, 2 mM dithiothreitol, 0.2% Triton X-100, and 50% glycerol was used throughout this study. Except where noted otherwise, reaction mixtures also contained the components of the storage buffer at the same dilution as the enzyme. A unit of reverse transcriptase activity is defined as the amount required to catalyze the incorporation of 1 nmol of TTP into acid-insoluble products in 10 min at 37 °C. (Reaction conditions are those specified by Life Sciences, Inc.)

When the experiments were designed to measure the production of cDNA in a given period, reactions were supplemented with either radioactive dGTP, dCTP, or dATP. When the aim was to study the rate of initiation of transcription, the 5′-end-labeled oligo(dT)$_{12-18}$ (see below) was substituted for its unlabeled counterpart. All reactions were monitored by determining the increase in trichloroacetic acid (Cl_3CCOOH)-precipitable radioactivity bound to GF/C glass fiber filters (Millipore, Bedford, MA) (Puskas et al., 1982). However, in order to avoid the high background and lack of precision associated with the use of the 5′-end-labeled primer, we diluted a 5-μL aliquot of each sample with 15 μL of buffer at pH 5.1 containing 8.3 mM sodium acetate, 2.8 mM EDTA, 42 mM NaCl, and 0.18 mg/mL nonradioactive oligo(dT)$_{12-18}$ as carrier. A measured aliquot of the diluted material was applied to the filter and quantified with a scintillation counter.

The size of the cDNA products was evaluated by diluting the samples with 10 mM sodium acetate at pH 5.1 containing 3 mM EDTA and 50 mM NaCl (ACE), extracting the nucleic acids with phenol, precipitating them in the presence of 10 μg of carrier tRNA, hydrolyzing the RNA in 0.1 N NaOH, and subjecting the DNA to electrophoretic analysis on 6% polyacrylamide–urea gels (Puskas et al., 1982). Radioactivity was visualized with X-Omat XR-5 film or a closely related equivalent. The band corresponding to full-length cDNA could be excised from the gel and quantified by measuring Cerenkov radiation (Puskas et al., 1982).

Determination of Ribonuclease H Activity. RNase H activity was measured in two ways. Qualitative assays were performed by treating 30 μg/mL globin mRNA with 117 units/mL reverse transcriptase and 5 μg/mL oligo(dT)$_{12-18}$ in the absence of deoxyribonucleoside triphosphates under the conditions described for cDNA synthesis; the reactions were terminated by extracting with phenol and precipitating the mRNA together with carrier tRNA. The products were analyzed electrophoretically on 6% polyacrylamide–urea gels and visualized by staining with ethidium bromide (Puskas et al., 1982). In quantitative assays of RNase H activity, cleavage of the poly(A) tail of the substrate, 20 μg/mL 3′-end-labeled globin mRNA, under the same conditions was evaluated. Radioactivity in intact globin mRNA could be precipitated with Cl_3CCOOH, whereas tracer in oligomeric products could not. The preparation of the end-labeled material is detailed below. In some cases, 1.33 mM each of dCTP, dGTP, and dATP was included in the reaction. Acid-insoluble RNA was collected on GF/C glass fiber filters as specified for reverse transcriptase products.

Preparation of 5′-^{32}P-End-Labeled Oligo(dT). In a volume of 10 μL, 10 μg of 5′-dephospho-oligo(dT)$_{12-18}$ was incubated at 37 °C for 20 min with 200 μCi of [γ-^{32}P]ATP and 100 units/mL polynucleotide kinase in 66 mM Tris-HCl buffer at pH 7.6 supplemented with 1 mM dithiothreitol and 10 mM $MgCl_2$. The mixture was brought to 100 μL with ACE buffer and extracted with phenol. Radioactivity in oligo(dT) was assayed by spotting appropriate aliquots on Whatman DE-81 filters (Arthur Thomas, Philadelphia, PA), drying them, and eluting unreacted [γ-^{32}P]ATP with 0.5 M sodium phosphate at pH 6.8. To remove residual ATP preparatively, we chromatographed the entire aqueous sample, 226 μL, on a prepacked PD-10, G-25M column (Pharmacia, Uppsala, Sweden) equilibrated and developed with water; fractions of 0.5 mL were collected. Samples containing oligo(dT), based on radioactivity measurements and the absorbance at 260 nm, were combined and reduced in volume. The specific activity was calculated from the chemical yield, which was virtually 100%, and the amount of ^{32}P incorporated. A value of 12×10^6 dpm/μg was obtained. Approximately one out of a thousand molecules was labeled.

Preparation of End-Labeled [^{32}P]Poly(A+) mRNA. Tracer was introduced into 5 μg of globin mRNA by extending the poly(A) tail with 10 units/mL poly(A) polymerase and 6 μCi of [α-^{32}P]ATP in a volume of 100 μL under previously described conditions (Puskas et al., 1982). The reaction was carried out at 37 °C for 20 min. The sample was extracted with phenol and subjected to column chromatography on G-25 Sephadex as detailed earlier for oligo(dT). Cl_3CCOOH-insoluble radioactivity in the starting sample and the eluted fractions was measured on GF/C filters. A peak of radio-

activity at the front contained globin mRNA. The relevant fractions were combined and precipitated with ethanol in the absence of carrier for 48 h at −20 °C. RNA was recovered by centrifugation at 27000*g* for 30 min. Severe losses occurred during the precipitation step, resulting in a yield of approximately 50%. The specific activity of the product was 5×10^5 dpm/μg. It was further diluted with nonradioactive globin mRNA to obtain the desired concentration.

The 3′-end-labeled mRNA was characterized by gel electrophoresis on 6% polyacrylamide–urea gels (see Figure 4). The analysis showed that the size distribution of radioactive globin mRNA was indistinguishable from that of unlabeled globin mRNA; i.e., the reaction did not result in a few greatly elongated and therefore highly radioactive molecules. The 3′-end-labeled molecules were also characterized on denaturing formaldehyde gels (Rave et al., 1979) and were found to be intact. Under the conditions used, approximately 2% of the molecules reacted, assuming that only one [^{32}P]adenylate group resided in each radioactive mRNA.

Determination of mRNA Levels after Synthesis of Single-Stranded cDNA. The methods described here were designed to measure the amount of translatable mRNA remaining after transcription by determining the ability of the sample to direct protein synthesis. Reverse transcription of mRNA was carried out in the absence of radioactive materials as described in individual experiments and elsewhere under Materials and Methods. Reactions were intiated by adding enzyme and warming to 37 °C. They were terminated at the stated times by transferring 25 μL (an amount corresponding to 0.5 μg of input globin mRNA) into 175 μL of ACE buffer (Puskas et al., 1982) and freezing in dry ice. Zero-time samples were removed immediately after the addition of enzyme to the cold reaction mix. All samples in an experiment were extracted with phenol as a group, and the resultant nucleic acids were precipitated in the presence of 10 μg of carrier tRNA. Three controls were performed: The 60-min control measured artifactual degradation of mRNA as a result of exposure to putative contaminants in the reverse transcriptase preparation; oligo(dT) and dNTPs were omitted. The minus mRNA control measured protein synthesis resulting from transcription reaction mixtures lacking template. Finally, to ensure that reverse transcriptase had actually synthesized cDNA, a 12.5-μL aliquot of the complete reaction mixture, which was devoid of radioactivity, was transferred to a separate vessel containing 2 μCi of dCTP or dGTP. Cl_3CCOOH-insoluble products were quantified on filters.

In order to determine the amount of mRNA in the samples, it was necessary to demonstrate a linear dose–response relationship between mRNA available and protein produced in response to it. For this purpose, varying amounts of mRNA were mixed with reverse transcriptase and buffer and subjected immediately to the entire procedure for sample preparation. These mRNAs were then used to generate a standard translation curve. It should be noted that the standard samples, the controls, and the test samples all contained tRNA; because tRNA inhibits translation, the amount included in samples which were to be compared was necessarily maintained constant.

Translation was carried out in a wheat germ system at room temperature in a volume of 30 μL according to the directions supplied by BRL. [^{35}S]Methionine at approximately 0.3 mCi/mL was used to label the synthesized proteins. Reactions were followed by spotting small aliquots onto Whatman 3MM filters and dropping them immediately into 10% Cl_3CCOOH containing 2 mg/mL DL-methionine. When all samples in a given set had been collected, the filters were boiled for 10 min to discharge aminoacyl-tRNA, washed in 5% Cl_3CCOOH, rinsed in ethanol, dried, and assayed in a scintillation counter. Based on the data obtained by translating the mRNAs that comprised the standards, we dissolved all nucleic acid precipitates from the test samples in 15 μL of H_2O and used 5 μL, an amount corresponding to 0.17 μg of input mRNA, for cell-free protein synthesis. Controls were treated similarly. Our results then lay within the linear range of the standard curve.

Gel Electrophoresis of Proteins. Electrophoresis of proteins in the presence of sodium dodecyl sulfate was carried out by the method of King & Laemmli (1971) with acrylamide concentrations of 3% and 12.5%, respectively, for the stacking and separating gels. Gels were stained, destained, and dried in a Bio-Rad (Richmond, CA) gel drier. ^{35}S-Labeled proteins were detected after 2–6 days of exposure to Kodak XR-5 or NS-2T no-screen X-ray film.

Kinetics. The rate of deadenylation of 3′-end-labeled globin mRNA by 28–280 units/mL reverse transcriptase was measured with 20 μg/mL globin mRNA (specific activity 2.1×10^4 dpm/μg) and 5 μg/mL oligo(dT)$_{12-18}$ in the absence of dNTPs. Samples also contained fixed quantities of the following enzyme diluents: 24 mM potassium phosphate at pH 7.2; 0.24 mM dithiothreitol; 0.024% Triton X-100; 6% glycerol. Initial rates were obtained by measuring the disappearance of acid-insoluble radioactivity in 5-μL aliquots at intervals of 30 s. Velocities were corrected for the presence of acid-insoluble radioactive nonsubstrates and products.

The rate of initiation of transcription was measured with a 0.5 mM sample of each dNTP, 20 μg/mL globin mRNA, 5 μg/mL 5′-end-labeled oligo(dT) (specific activity 9.8×10^6 dpm/μg), 23–130 units/mL enzyme, and the same concentrations of diluents. Initial rates were obtained by determining the appearance of Cl_3CCOOH-insoluble radioactivity in 5-μL aliquots at intervals of 30 s. This approach does not strictly measure initiation because, on the average, several nucleotides must be added per primer to render it acid insoluble. However, since the elongation reaction is known to be processive (Gregerson et al., 1980), these events follow in close succession. A molecular weight of 4500 for oligo(dT)$_{12-18}$ was used when converting micrograms to micromoles.

Results and Discussion

Effect of Reverse Transcriptase Concentration on the Yield of Full-Length Single-Stranded cDNA. Transcription of globin mRNA was studied at three concentrations of reverse transcriptase spanning 2 orders of magnitude. Aliquots of the total reaction mixture were removed throughout a 60-min incubation and subjected to gel electrophoresis in order to determine the size of the products and the amount of full-length single-stranded cDNA produced. Figure 1 shows the kinetics of synthesis of completed cDNA molecules as a function of enzyme concentration. By inspection, it can be seen that the amount of the full-size product is strongly dependent on the enzyme levels. At a very low concentration of reverse transcriptase, 11.7 units/mL, virtually no full-length cDNA was observed. At an intermediate concentration, 117 units/mL, full-length cDNA was produced, but by increasing the reverse transcriptase concentration from 117 to 1170 units/mL, a 10-fold increase in the mature product was observed after 60 min of reaction. The band of completed cDNA was by far the darkest on the autoradiogram of the gel. The molar yield of mature cDNA relative to input mRNA was 19% after 1 h of incubation of globin mRNA with the highest enzyme concentration; approximately 30% of the total cDNA

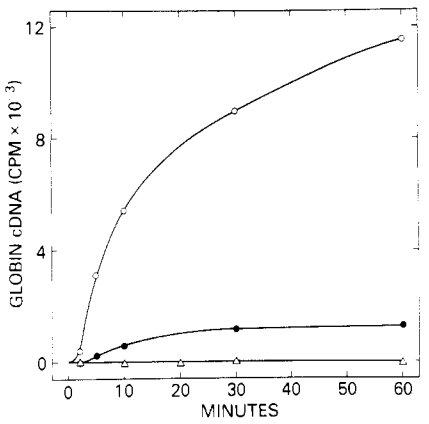

FIGURE 1: Effect of reverse transcriptase concentration on the yield of single-stranded full-length globin cDNA. Synthesis of cDNA was carried out with 35 μM of each deoxyribonucleoside triphosphate. The labeled substrate was [^{32}P]dATP. All samples contained 20 mM potassium phosphate, at pH 7.2, 0.2 mM dithiothreitol, 0.02% Triton X-100, and 5% glycerol. At the stated times, 50 μL from each reaction mixture was used for acrylamide gel electrophoresis. The area of the gel corresponding to full-length cDNA was excised and quantified by monitoring the Cerenkov radiation. Reverse transcriptase concentrations were 1170 (○), 117 (●), and 11.7 units/mL (△).

Table I: Why Does Reverse Transcriptase Stop Synthesizing cDNA?[a]

	full-length cDNA synthesis		
additions at 60 min	time (min)	cDNA (cpm)	(cDNA in 120 min)/ (cDNA in 60 min)
none	60	24 800	
none	120	26 200	1.06
reverse transcriptase	120	25 200	1.02
globin mRNA	120	35 900	1.45
globin mRNA and reverse transcriptase	120	43 100	1.74

[a] Globin cDNA was synthesized with 117 units/mL reverse transcriptase in a reaction mixture containing 20 μg/mL globin mRNA, 5 μg/mL oligo(dT)$_{12-18}$, 35 μM of each deoxyribonucleoside triphosphate, [^{32}P]dATP at ~30 μCi/mL, and the standard concentrations of buffer and salts. After 60 min at 37 °C, the sample was divided into five equal aliquots. Where indicated, the concentrations of globin mRNA and reverse transcriptase were doubled relative to zero-time values. Incubation of four of the five aliquots, as noted, was carried out at 37 °C for an additional 60 min. The fifth sample was frozen. At 2 h, an aliquot of each sample corresponding to 40 μL of the reaction mix at the commencement of the experiment was analyzed electrophoretically. Radioactivity incorporated into full-length cDNA was determined by excising the appropriate band from the gel. The data are presented as the ratio of labeled full-length cDNA produced in 120 min to that produced at 60 min.

was full length. Prolongation of the reaction beyond 60 min resulted in little or no increase in yield (see Tables I and II).

The time required for synthesis of full-length cDNA can also be inferred from the data. Completed molecules can be visualized on gels after 2 min of reaction although, on the basis of the initial rate of appearance of the mature product, the actual time required for reverse transcriptase to traverse globin mRNA was between 1 and 1.5 min. Presumably, cDNA molecules first appearing at 60 min of incubation of mRNA with the enzyme were initiated between 58 and 59 min. Clearly, initiation is rate limiting.

The dependence of the yield of cDNA at completion on the enzyme concentration suggests that interfering reactions have occurred. Without secondary reactions, the enzyme concentration would determine the rate of reaction but not the yield

Table II: Effect of Deoxyribonucleoside Triphosphates on the Yield of cDNA[a]

	full-length globin cDNA, % yield	
[dNTP] (mM)	60 min	120 min
0.035	6–7	8
0.175	16	19
0.50	21	22
1.0	24	26

[a] Globin mRNA at 20 μg/mL was transcribed with 117 units/mL reverse transcriptase in the presence of 5 μg/mL oligo(dT)$_{12-18}$, ~200 μCi/mL [^{32}P]dCTP, and the stated concentration of each deoxyribonucleoside triphosphate. At 60 min, the concentration of reverse transcriptase was doubled relative to zero-time values in those samples which were not terminated. Products in 50-μL aliquots of the reaction mix were prepared for electrophoresis after 60 or 120 min of incubation and separated in 6% polyacrylamide-urea gels. The percent yield of full-length single-stranded cDNA, defined as (moles of full-length cDNA per mole of mRNA) × 100, was determined by excising the appropriate band from a gel and measuring the Cerenkov radiation. In making the calculations, it was assumed that the sequence of deadenylated globin mRNA was 25% guanylic acid residues.

of product at completion. With reduced enzyme levels, it should simply take longer to reach the same end point. In the following section, we will rule out trivial explanations of our results such as thermal denaturation of reverse transcriptase, depletion of one or more triphosphates, or degradation of substrates by contaminants. We will show that the obstacle to higher cDNA yields resides in a competing reaction catalyzed by reverse transcriptase.

Why Did the Synthesis of cDNA Stop? A survey of the macromolecules in the reaction mixture was undertaken to determine which component of the reverse transcriptase reaction became exhausted when further synthesis of cDNA was halted. After 60 min of catalysis with intermediate levels of enzyme and with the dNTP concentration near the K_m value, the reaction mixture was divided into five identical portions. One was frozen immediately. To the others were added, respectively, nothing, fresh reverse transcriptase, globin mRNA, and both globin mRNA and reverse transcriptase. An additional hour of incubation was allowed before all samples were extracted with phenol and analyzed electrophoretically. The results (Table I) showed that the addition of fresh enzyme did not stimulate synthesis of significant quantities of new cDNA. In contrast, the addition of globin mRNA after 60 min resulted in a marked increase in full-length cDNA, whereas a dramatic increase in yield accompanied the addition of both enzyme and mRNA. Despite some loss of enzyme activity, globin mRNA was the only component which, alone, could reinitiate transcription. The synthesis of cDNA had stopped because the globin mRNA had been consumed.

The data rule out the possibility that phosphatases, introduced as artifacts, degraded dNTPs during the first 60 min of reaction. The dNTP levels, which were 35 μM at the beginning of the experiment, were evidently sufficient to support further cDNA synthesis in the second hour.

The possibility that contaminating ribonuclease in the reverse transcriptase preparation was responsible for the disappearance of mRNA was subsequently investigated. As an assay for the integrity of the mRNA, we measured its ability to serve as a template for synthesis of full-length cDNA after exposure to the enzyme in the presence and absence of oligo(dT) at 37 °C. After 60 min of incubation with putative ribonucleases, the missing ingredients for the complete RNA-directed DNA polymerase reaction, together with fresh

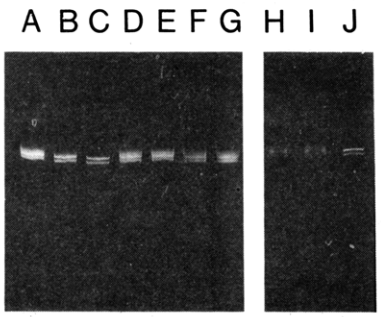

FIGURE 2: Effect of reverse transcriptase and oligo(dT) on the size of globin mRNA. Globin mRNA at 30 μg/mL was treated with 117 units/mL reverse transcriptase in the presence and absence of oligo(dT). Triphosphates were omitted. Aliquots of 50 μL were removed and prepared for electrophoretic analysis. Lanes A, B, and C are 0-, 10-, and 60-min incubations, respectively, with oligo(dT); lanes D, E, and F are 0-, 10-, and 60-min incubations, respectively, without oligo(dT). Lanes G and H are 1.5 and 1 μg, respectively, of untreated globin mRNA exposed neither to any of the components required for cDNA synthesis nor to phenol. Lanes I and J are 50 μL of 20 μg/mL globin mRNA incubated for 60 min with oligo(dT) in the absence and presence of reverse transcriptase, respectively.

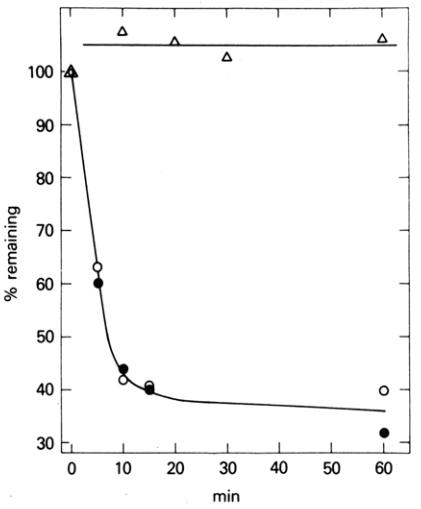

FIGURE 3: Deadenylation of globin mRNA by reverse transcriptase. Globin mRNA labeled at the 3′ end was mixed with unlabeled globin mRNA (final specific activity 6.3×10^4 dpm/μg, final concentration 20 μg/mL) and treated with 117 units/mL reverse transcriptase and 5 μg/mL oligo(dT)$_{12-18}$. Where indicated, dGTP, dCTP, and dATP each at 1.33 mM were also included. At the stated times, aliquots in duplicate were removed for analysis on filters. Numbers on the ordinate represent the average amount of trichloroacetic acid precipitable radioactivity remaining in mRNA expressed as a percentage of the zero-time value. The average amount of radioactivity in acid-precipitable mRNA in a 2-μL aliquot at zero time was 2500 dpm. (○) Deadenylation in the absence of dNTPs; (●) deadenylation in the presence of three dNTPs; (△) globin mRNA treated with reverse transcriptase under the conditions noted above in the absence of both oligo(dT) and triphosphates.

enzyme, were added. The results after 60 min of reaction indicated that a yield of mature cDNA of ~7% had been achieved despite pretreatment of mRNA with reverse transcriptase. This value compares favorably with the amount of cDNA produced under the same conditions without pretreatment (Table II, 35 μM dNTPs). Messenger RNA had apparently survived. However, when oligo(dT) was also included during the first incubation, the yield of mature cDNA, measured later, decreased to 3.5% (data not shown). The results suggested that the factor responsible for the destruction of mRNA was either partly or fully dependent on the presence of oligo(dT).

Effect of Oligo(dT) and Reverse Transcriptase on the Size of Globin mRNA. Globin mRNA was incubated at 37 °C with reverse transcriptase and oligo(dT)$_{12-18}$, where indicated, to provoke degradation of the mRNA. Samples treated with reverse transcriptase in the absence of oligo(dT) remained virtually unchanged throughout 60 min of exposure (Figure 2). The globin mRNA appeared as a diffuse band on the gel owing to heterogeneity of poly(A) tails (Sheiness & Darnell, 1973). However, when oligo(dT) was included, the broad band of mRNA viewed at zero time disappeared, to be replaced in 60 min by two very sharp, slightly smaller bands. Incubation of the mRNA with oligo(dT) in the absence of enzyme had no effect on the size. Hence, contaminating ribonuclease in the primer preparation was ruled out. Since rabbit globin mRNA is known to be a mixture of the mRNAs for the α and β subunits, both of which are polyadenylated, these results are consistent with deadenylation as a result of hybridization of oligo(dT) to poly(A) regions in both molecules. Although such deadenylation reactions are known to occur with RNase H from other sources (Vournakis et al., 1975; Donis-Keller, 1979), the ability of reverse transcriptase to accomplish the same task had not previously been suspected.

Quantitative Evaluation of Ribonuclease H Activity. In order to show that the shortening reaction actually removed poly(A) tails, we developed a quantitative assay which made use of 3′-end-labeled mRNA as a substrate. Figure 3 shows that reverse transcriptase rapidly removed the 3′-terminal portion of globin mRNA only when oligo(dT) was included in the reaction. The kinetics were equivalent to those observed qualitatively in the gel assay of Figure 2; in 10 min, the reaction did not quite go to completion. The data also demonstrate that deoxyribonucleoside triphosphates had no effect on deadenylation. By omission of TTP and inclusion of the three remaining dNTPs, cDNA synthesis was prohibited while high levels of triphosphates were maintained. In contrast, the rate of deadenylation was a function of primer length; with oligo(dT)$_{17}$ at 5 μg/mL, the initial rate was ~10-fold higher than with oligo(dT)$_8$ at the same concentration (data not presented). Taken together, these data show that the reaction that shortens and resolves α- and β-globin mRNAs (Figure 2) does so by removing the 3′ ends of the molecules.

The sizes of the 3′-end-labeled mRNA substrate and the products after 60 min of reaction with reverse transcriptase in the presence of oligo(dT) were investigated by slab gel electrophoresis. The results in Figure 4 indicated that the 3′-end-labeled substrate was the same size as unlabeled globin mRNA and that virtually all the radioactivity found in the products, some of which were Cl_3CCOOH soluble, was less than 40 nucleotides long. The small amount of radioactivity in large molecules probably came from the addition of one or a few adenylate residues to poly(A)-negative contaminants such as 18S rRNA or fragments derived from it.

Translation of Previously Transcribed Globin mRNA. In order to show that deadenylation of mRNA *during* synthesis of cDNA was responsible for the loss of template activity, we investigated three sets of transcription conditions resulting in differing yields of completed cDNA. In each case, the quality of the mRNA was evaluated by translation. Under the least favorable conditions for cDNA synthesis (low enzyme concentration and low dNTP concentration), approximately 48% of the input mRNA remained after transcription had taken place for 1 h (Table III), whereas 6–7% was converted to full-length cDNA. When the concentration of dNTPs was increased to 1 mM, cDNA yields increased to 24% (Table II), whereas translatable mRNA was reduced to approximately 25% of the total. Under optimized conditions with mature cDNA yields approaching 50% in 1 h (Puskas et al., 1982),

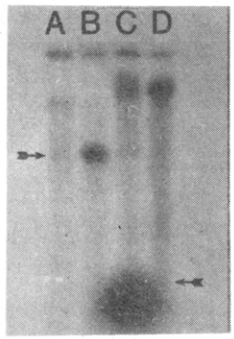

FIGURE 4: Size of 3′-end-labeled globin mRNA and of the products of ribonuclease H activity. Globin mRNA labeled at the 3′ terminus was mixed with unlabeled globin mRNA (final specific activity 6.0 × 10^4 dpm/μg, final concentration 20 μg/mL) and treated with reverse transcriptase and oligo(dT) for 60 min as in Figure 3. The products from several samples containing three deoxyribonucleoside triphosphates varying from 0.2 to 4 mM in total triphosphate concentration were mixed, diluted with an equal volume of 0.1% dye markers and 10 M urea, and analyzed on a 6% polyacrylamide–urea gel. (Samples were not extracted with phenol or precipitated with ethanol.) Control globin mRNA was incubated for 60 min with reverse transcriptase in the absence of oligo(dT) and triphosphates under the same conditions. It was subsequently diluted with 1 mM Tris-HCl at pH 8.0 containing 50 mM NaCl in order to equalize the acid-insoluble radioactivity and the salt in both samples. An aliquot corresponding to 10 000 dpm of either acid-precipitable products or control globin mRNA was applied to each lane of the gel. Lanes A and B, products and control globin mRNA, respectively, run for 3.5 h at 200 V. Lanes C and D, products and control globin mRNA, respectively, run for 1 h under the same conditions. The *left* arrow marks the position of unlabeled globin mRNA run for 3.5 h stained with ethidium bromide. The *right* arrow marks the position of bromphenol blue, which comigrates with oligo$(A)_{29-30}$, run for 1 h.

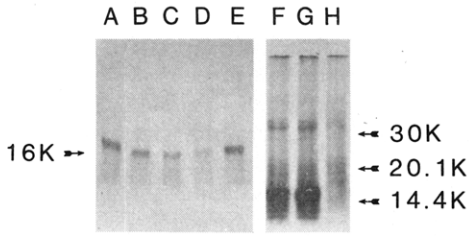

FIGURE 5: Analysis of the translation products of previously transcribed globin mRNA. Globin mRNA was transcribed with 117 units/mL reverse transcriptase under the conditions in Table II with either a 35 μM or a 1 mM sample of each deoxyribonucleoside triphosphate and translated for 60 min. In each case, the products from two-thirds of the total wheat germ assay, i.e., 20 μL, were analyzed in polyacrylamide gels. Lanes A–D, translation products after transcription with a 35 μM sample of each dNTP for 0, 15, 30, and 60 min, respectively; lane E, translation products from the 60-min control, i.e., mRNA incubated with reverse transcriptase in the absence of both dNTPs and oligo(dT); lane F, translation products after transcription with a 1 mM sample of each dNTP for 30 min (70% recovery of acid-precipitable peptides relative to the 60-min control); lane G, 60-min control corresponding to the sample in lane F; lane H, wheat germ translation products with a 1 mM sample of each dNTP in the absence of exogenous mRNA. Arrows indicate the location and molecular weights of unlabeled protein standards.

no mRNA activity was detected relative to controls run in the absence of added mRNA.

In these experiments, we assumed that acid-precipitable ^{35}S was a direct reflection of functional mRNA activity. To support this view, the size of the protein products was determined electrophoretically. Although bands other than globin appeared on the gel (Figure 5), a 16 000-dalton protein, the correct size for globin, was the dominant product synthesized in response to the suviving RNA molecules. Furthermore, the amount of both globin protein and acid-precipitable labeled peptides diminished with increasing time of transcription

Table III: Translation of Previously Transcribed Globin mRNA[a]

transcription			translation of acid-precipitable peptides	
[enzyme] (units/mL)	[dNTP] (mM)	time (min)	cpm	% of zero time of transcription[b]
117	0.035	0	11 000	100
117	0.035	5	9 700	84
117	0.035	15	7 500	57
117	0.035	30	6 700	46
117	0.035	60	6 200	40
117	0	60	9 700[c]	84
117	1.0	0	62 300	100
117	1.0	60	39 200	29
117	1.0	120	38 600	27
117	1.0	0	76 000	100
117	1.0	30	64 900	76
117	1.0	60	38 000	18
117	0	60	72 000[c]	92
585	0.5	0	16 300	100
585	0.5	10	7 600	20
585	0.5	30	4 400	0
585	0.5	60	3 400	0
585	0	60	16 800[c]	106

[a] Globin mRNA at 20 μg/mL was transcribed with reverse transcriptase at the stated concentration at 37 °C. Samples contained, in addition, 5 μg/mL oligo$(dT)_{12-18}$ and the buffer and salts specified elsewhere. At the stated times, aliquots were harvested. Subsequently, the mRNA together with cDNA and carrier tRNA was translated in the wheat germ system for 60 min. Appropriate controls as detailed under Materials and Methods were included. The amount of [^{35}S]methionine incorporated into trichloroacetic acid precipitable peptides is presented without correction. [b] These values represent the yield of protein synthesized in response to surviving mRNA in the test sample relative to that synthesized in the presence of mRNA harvested at zero time of transcription. Before the results were expressed as a percent, protein synthesis directed by endogenous wheat germ mRNA adulterated with carrier tRNA was subtracted from both samples. [c] 60-min control; i.e., oligo(dT) and dNTPs were omitted.

(Table III). In the absence of added nucleic acids, no globin was observed.

The data show that fully functional mRNA remained in the reaction mixture after templates for cDNA synthesis had vanished and that the sum of full-length cDNA and deadenylated mRNA accounted for only half of the input mRNA.

Comparison of the Rate of Deadenylation and the Rate of Initiation of Transcription. Having established the existence of competing reactions, we examined the kinetics of each with the aim of predicting yields of both cDNA and deadenylated mRNA. Both the initial rate of initiation of polymerization and the initial rate of deadenylation were linear functions of the enzyme concentration with slopes of 0.55 and 0.14 pmol min^{-1} (unit of enzyme)$^{-1}$, respectively. Since the curves intersected each other at the origin, the rate of initiation of polymerization was 4-fold higher at any enzyme concentration than that of deadenylation. Furthermore, reciprocal plots of the same data, namely, $1/v$ vs. $1/[E]$, revealed that there was neither a maximum velocity nor an apparent K_m value for either reaction under the conditions used; again, both curves passed through the origin.

From these results, one would predict that a change in the ratio of the rates of the two activities catalyzed by reverse transcriptase would affect the yields of full-length cDNA. Such a change can be effected by varying the dNTP concentration at a fixed enzyme concentration. As shown in Table II, with 117 units/mL reverse transcriptase, the yield of full-length cDNA produced in 1 h increased from approximately 7% at 35 μM dNTPs, slightly higher than the K_m value of 25 μM (Margalith et al., 1982), to 24% when the dNTP

Table IV: Effect of Radioactive dCTP and TTP on Single-Stranded cDNA Synthesis[a]

labeled precursor	dpm × 10^{-3}	
	Cl_3CCOOH-precipitable cDNA	full-length cDNA
dCTP	119	30.7
TTP	160	30.8

[a] Globin cDNA was synthesized at 37 °C for 1 h with 60 units/mL reverse transcriptase and either dCTP or TTP at 35 μM. The specific activities were 580 and 650 μCi/μmol, respectively. The data obtained with dCTP have been normalized accordingly.

concentration was no longer limiting.

Although the dependence of yield on the dNTP concentration was consistent with the characteristics of the competing reactions catalyzed by reverse transcriptase, the dependence of yield on enzyme concentration described in Figure 1 was inconsistent; the ratio of the intial rates for the two reactions was constant *regardless* of enzyme concentration. Clearly, the kinetics do not explain observed cDNA yields.

Development of a Model. It is puzzling that the quantitative aspects of the two reactions catalyzed by reverse transcriptase, RNase H activity and polymerase activity, cannot be used to predict cDNA yields. The rate of initiation of polymerization at saturating levels of dNTPs is 4-fold higher than the rate of deadenylation. Hence, 75% of the input mRNA might be expected to be converted to full-length cDNA. This is clearly not the case under any conditions that have been investigated. The problem stems from equating the rate of initiation of cDNA synthesis with the yield of cDNA. A simple comparison makes the point. At an enzyme concentration of 117 units/mL, the rate of initiation of cDNA synthesis from kinetic considerations was ~66 pmol min^{-1} mL^{-1} at saturating dNTP levels or, by calculation, ~33 pmol min^{-1} mL^{-1} at the K_m value. At 35 μM dNTPs, the yield in 1 h for the same enzyme concentration was 6–7% (Table II) or 6–7 pmol mL^{-1}. In short, the number of cDNA molecules initiated *in the first minute* of the reaction exceeded the number of completed cDNA molecules produced *in 60 min* by 5-fold. The data show that most oligo(dT) molecules that were extended were aborted soon afterward.

The existence of abortive initiation is further borne out by the data in Table IV: Cl_3CCOOH-precipitable radioactivity is 1.3-fold higher when TTP instead of dCTP is used as the labeled precursor, but the amount of radioactivity incorporated into full-length cDNA is virtually identical regardless of which triphosphate is radioactive.

We believe that abortive initiation occurs because many oligo(dT) primers can bind to the poly(A) tail of a single messenger RNA molecule and be lengthened but only one primer, that bound closest to the junction of the poly(A) tail with the 3′-noncoding region (hereafter called the critical junction), can give rise to full-length cDNA. All others are obstructed by moieties located downstream, toward the 5′ end of mRNA. This view predicts that long stretches of 3′-terminal poly(dA) in cDNA will be rare, as borne out by the cloning of γ-interferon (Gray et al., 1982).

It would appear from this analysis that the yield of cDNA must be determined by the rate of "useful" or "productive" initiation relative to the rate of deadenylation. In our kinetic studies, we compared the rate of initiating cDNA synthesis on several primer molecules per poly(A) tail with the rate of removing the 3′-terminal ribonucleotide. For prediction of the yield of mature cDNA, the ratio of the rate of initiation of polymerization to that of clevage of hybridized RNA must be ascertained for a single primer. We suspect that this ratio is approximately unity for two reasons: (1) Under conditions in which an average of three primers were able to bind to one poly(A) tail, we found 4-fold more intiation of polymerization than clipping of the 3′ terminus of the RNA; hence, the rate of initiation per primer should be one-third that measured. Since the observed rate of deadenylation probably adequately reflects the events at a single primer, the probability of polymerization would be ~1.3-fold higher than that of deadenylation. (2) The yields of cDNA under optimized conditions approach 50% (Puskas et al., 1982). If deadenylation were the only means of limiting production of intact cDNA, the maximum yield of full-length cDNA should be 57% (57% transcribed/43% deadenylated = 1.3), an estimate slightly higher than observed values. However, as we will show below, other mechanisms evidently exist for diminishing the yield.

The events on the poly(A) tail must be examined in more detail in order to understand the dependence of the yield of cDNA on the concentration of enzyme when the kinetics would suggest otherwise. At high enzyme concentration, every primer–RNA hybrid will be either cleaved or extended, depending solely on the ratio of the velocities of the two reactions. However, only the fate of the primer nearest the critical junction will dictate the yield of mature cDNA; identical events occurring upstream, virtually simultaneously, will be irrelevant. At a low enzyme concentration, ≤1 primer–RNA hybrid per poly(A) tail, early in the reaction, can serve as a catalytic site; the position of the primer involved is crucial. If the primer nearest the critical junction is the target, the same analysis can be applied. However, with catalytic quantities of enzyme and several primers per tail, other targets are favored statistically. Focusing on primers located away from the critical junction, we find that the same ratio of rates of the two reactions leads to different results; i.e., regardless of whether the primer is extended or the RNA is cleaved, it is unlikely that mature cDNA will be produced. Any primer lying nearer to the critical junction can block the synthetic reaction. Instead of generating a full-length cDNA, the polymerization reaction will produce a longer oligo(dT) primer which is in turn a better substrate for RNase H. The poly(A) tail will be shortened because there are no constraints on cleavage. Once RNA is severed, initiation upstream becomes nonproductive. When the size of the oligo(A) is reduced sufficiently to approximate that of the primer, the rate of polymerization would be expected to decline. Statistically, half the hybridized oligo(dT) molecules would present 3′-unhybridized ends to reverse transcriptase, a conformation that precludes polymerization but remains fully compatible with the requirements of RNase H. Under these conditions the rate of deadenylation will exceed that of initiation of polymerization. The net result expected is a decrease in yield of completed cDNA at low enzyme concentration, an expectation with which the data concur.

It would appear that oligo(U) primers could circumvent the problems generated by the RNase H activity. Antithetically, experiments with $oligo(U)_{10-30}$ showed that yields of full-length cDNA after 2 h were ~10-fold lower than with $oligo(dT)_{12-18}$.

Another puzzling phenomenon is the inability to account for all the mRNA that entered the reverse transcriptase reaction. We propose that the RNase H activity is responsible for the destruction of RNA, rendering it neither translatable nor transcribable with oligothymidylate. We suggest that the substrate for this reaction is a cDNA–RNA hybrid in which an oligoribonucleotide bound internally to mRNA serves as a primer for incorporation of deoxyribonucleotides. During

cDNA synthesis, the RNase H activity cleaves the mRNA. Later in the reaction, it can be shown that surviving fragments of bound RNA prime the synthesis of the second strand (Watson et al., 1979; Myers et al., 1980). From the availability of bare tracts of first-strand cDNA able to act as templates for second-strand synthesis, it can be inferred that some mRNA fragments, initially bound, have been released into solution. These oligoribonucleotides together with oligoadenylate derived from the deadenylation reaction comprise the available pool of RNA primers. We think that, in a manner analogous to calf thymus DNA random primers (Taylor et al., 1976), these molecules can prime the synthesis of the first strand of cDNA by binding to the body of deadenylated mRNA. Such a reaction explains the failure to find poly(A)-free (translatable) globin mRNA in optimized transcription reactions. The absence of such molecules cannot be ascribed to premature termination of cDNA. However, both truncated cDNAs as well as internally primed transcripts can reduce mature cDNA yields below the levels dictated by the relative rates of polymerization and deadenylation. Clearly, the inhibition of RNase H and/or the development of reverse transcriptases without such activity are worthwhile aims from a practical standpoint. Although fluoride or pyrophosphate has been advocated for this purpose (Brewer & Wells, 1974; Myers & Spiegelman, 1978), both putative inhibitors of the associated RNase H have now been repudiated (Srivastava et al., 1981).

Registry No. Oligo(dT), 25086-81-1; RNase H, 9050-76-4; reverse transcriptase, 9068-38-6.

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