Enzymatic Synthesis of Deoxyribonucleic Acid by the Avian Retrovirus Reverse Transcriptase in Vitro: Optimum Conditions Required for Transcription of Large Ribonucleic Acid Templates†

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ABSTRACT: In this communication, we present data which describe optimum conditions for reverse transcription of large ribonucleic acid (RNA) templates into deoxyribonucleic acid (DNA) transcripts by the avian retrovirus reverse transcriptase in vitro. In contrast to previous studies, we have optimized all of the reaction components with respect to their influence on the size of DNA transcripts rather than the incorporation of radio-labeled deoxynucleoside triphosphates into acid-insoluble DNA product. The most dramatic effect on uninterrupted reverse transcription is the presence of physiological concentrations (i.e., 148 mM) of monovalent cation in the reaction mixture, although all of the components of the reaction influence the size of the DNA transcripts synthesized to some extent. The enzymatic conditions described herein for the uninterrupted reverse transcription of large RNA templates (>1000–2000 nucleotides) are superior to those described previously because they are reproducible, do not require the presence of ribonuclease inhibitors, and do not result in the precipitation of components of the reaction mixture during incubation.

The discovery of the reverse transcriptase not only provided information concerning the mode of replication of retroviruses but has also provided the means by which RNA species from virtually any source can be converted into DNA for use as specific probes in hybridization experiments and genetic cloning in bacteria. Numerous reports have been published describing the various enzymatic conditions required for the reverse transcription of RNA (Monahan et al., 1976; Efstratiadis et al., 1975; Hell et al., 1976; Buell et al., 1978). However, for the most part, these previously described conditions, although satisfactory for reverse transcription of small RNA molecules (≤2000 nucleotides in length), did not lend themselves to uninterrupted transcription of larger RNA templates (i.e., >2000 nucleotides in length). These previously reported reaction conditions also suffered from inefficient transcription, poor reproducibility of transcription, and reduced specific activities of resultant cDNA[1] probes. Although reaction conditions were also described that facilitated uninterrupted reverse transcription of very large RNA templates (i.e., 7500–10 000 nucleotides in length) (Kacian & Myers, 1976; Myers et al., 1977), it soon became apparent that these particular reaction conditions were not ideal for routine reverse transcription reactions since they contained components that resulted in the insolubilization of the reaction mixtures during incubation. In our hands, this phenomenon severely limited the reproducibility of both the efficiency of reverse transcription and the ability of the enzyme to transcribe large RNA templates into similar sized DNA transcripts. Moreover, the components added to enzymatic reactions to facilitate uninterrupted reverse transcription by presumably inhibiting residual RNase activity contaminating the purified reverse transcriptase preparations also inhibited the reverse transcriptase-associated RNase H activity, thereby reducing double-stranded DNA synthesis (Myers & Spiegelman, 1978).

Because of the difficulties inherent in the various reaction conditions previously published and the demand for optimum conditions of uninterrupted reverse transcription of large RNA molecules, we have performed a series of studies to elucidate reaction conditions required to reproducibly generate large cDNA copies of high molecular weight RNA species. In contrast to previous studies, this analysis was accomplished by determining the optimum conditions necessary for increasing the length of the DNA transcripts rather than by assaying for optimal incorporation of deoxynucleoside triphosphates into DNA product, which could reflect increased initiation of DNA synthesis, elongation, or rate of incorporation of deoxynucleoside triphosphates. In this report, we present data which indicate that elongation of DNA transcripts in reconstructed reactions is dependent upon optimal pH, divalent cation, monovalent cation, temperature, and deoxynucleoside triphosphate concentration. Moreover, we demonstrate that under a particular set of conditions, the majority of the DNA transcripts synthesized on high molecular weight (~7500 nucleotides) RNA·oligo(dT)$_{12-18}$ template·primer complexes are genome-length in size and represent the bulk of the nucleotide sequences of the RNA template employed. The most dramatic effect on uninterrupted DNA transcription involves the concentration of monovalent cation which must reflect physiological conditions for efficient transcription to occur.

Materials and Methods

Reagents. The sources and preparation of most of the materials used in this study have been previously described (Faras et al., 1972, 1974; Faras & Dibble, 1975). All reagents were prepared in RNase-free glass- or plasticware.

Purification of Viral RNA and Reverse Transcriptase. Viral 70S RNA was purified from AMV by previously described procedures (Faras & Dibble, 1975). The 35S viral RNA subunits, free of endogenous primer and other low molecular weight RNAs, were also prepared as previously described (Collett & Faras, 1977; Faras & Dibble, 1975). The

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[1] Abbreviations used: oligo(dT)$_{12-18}$, oligo(deoxythymidylic acid); tRNA^{Trp}, tryptophan transfer ribonucleic acid; AMV, avian myeloblastosis virus; dNTPs, deoxynucleoside triphosphates; RNase, ribonuclease; cDNA, complementary deoxyribonucleic acid.

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physical integrity of the 35S subunits was assayed before use by heat denaturation of a 1-μg sample (30–45 s at 100 °C), followed either by electrophoresis under denaturing conditions on 3.5% polyacrylamide and 98% formamide gels (see below), which were subsequently stained with ethidium bromide, destained, and observed visually for band homogeneity, or by band-forming velocity centrifugation in a Model E analytical ultracentrifuge equipped with UV optics, with the resulting photographs analyzed on a Joyce-Loebl Mark III microdensitometer.

AMV RNA-directed DNA polymerase was obtained from J. Beard, Life Sciences, Inc., St. Petersburg, FL, under the auspices of the Office of Resources and Logistics, Viral Cancer Program, National Cancer Institute. Since reverse transcriptase obtained from this source is only partially purified, further purification was required to remove residual RNase activity from the preparations. The enzyme was assayed for template nicking activity by incubating it with ^{32}P-labeled RSV 35S RNA under conditions employed for the enzymatic synthesis of DNA or in 0.10 M NaCl, 0.02 M Tris-HCl, pH 7.4, and 0.001 M Na_2EDTA (STE buffer). The RNA was then heat denatured as described above, applied to a 5–20% (w/w) sucrose–STE gradient, and sedimented in a SW 50.1 rotor at $234000g_{av}$ for 120 min at 20 °C. If significant deviations from the profile of control ^{32}P-labeled 35S RNA (unincubated or incubated at 41 °C) were observed, the enzyme was further purified by previously published procedures (Collett & Faras, 1975a,b; Faras et al., 1972). In our hands most, if not all, of the AMV RNA-directed DNA polymerase preparations required further purification, a requisite for uninterrupted synthesis of long DNA transcripts.

Iodination of dCTP. ^{125}I-Labeled dCTP was prepared by using a modification of the Commerford reaction (Commerford, 1971; Kamen et al., 1976). A 6.25-nmol amount of dCTP (P-L Biochemicals) was incubated with 3 mCi of ^{125}I (Amersham/Searle IMS 300, >350 mCi/mL, carrier free). Isotopic dCTP prepared in this manner had a specific activity of 250–325 Ci/mmol.

Enzymatic Synthesis of cDNA in Reconstructed Reactions. The standard conditions for synthesis of DNA in reconstructed reactions containing purified reverse transcriptase and RNA have been described (Collett & Faras, 1977). However, the optimal conditions utilized for the maximum synthesis of genome-length DNA transcripts are a modification of the published protocol. Briefly, the reaction mixture was prepared as follows, adding the components in order: 188 μL of 0.10 M Tris-HCl, pH 8.1 at 25 °C, 15 μL of 0.10 M $MgCl_2$, 25 μL of [^{125}I]dCTP (250–310 Ci/mmol), 2.5 μL of each dNTP at a concentration of 5 mg/mL, 6 μL of 2-mercaptoethanol; RNA and oligo(dT)$_{12-18}$ were added to a final concentration of 4 and 2 μg/mL, respectively. Purified $\alpha\beta$ DNA polymerase was added at 30–60 units/mL of reaction mixture. Finally, 2 M KCl (or NaCl) was added to the desired final concentration (optimally 148 mM). The reaction was allowed to proceed for 1 h at the temperatures indicated in the figure legends. Other conditions of enzymatic synthesis employed in these studies include "rate-limiting" and "millimolar" concentrations of dNTPs and are described in detail elsewhere (Collett & Faras, 1977). The principal difference between these reaction conditions resides in the concentration of dNTPs. "Rate-limiting" refers to a lower concentration of dCTP (7.5 μM) than that routinely employed in our standard reaction mixtures (75 μM), whereas "millimolar" indicates that all dNTPs are present at a concentration of 1 mM in the reaction. The inclusion of ribonucleoside triphosphates in the reaction was as described elsewhere (Clayman et al., 1979). Variations in the conventional reaction conditions to determine precise optima are indicated in the appropriate figure legends. Following enzymatic synthesis, reaction mixtures were deproteinized by treatment with sodium dodecyl sulfate ($NaDodSO_4$)–Pronase, phenol extracted, and subjected to either alkaline (0.3 M NaOH in STE, 4 h, 37 °C) or RNase (40 μg/mL, 45 min, 37 °C) hydrolysis to remove the template RNA. The cDNA samples were then ethanol precipitated for concentration, pelleted, and resuspended in formamide prior to analysis. Because of the recent report (Masamune & Richardson, 1977) demonstrating the presence of oligo(rGTP) contaminating commercial preparations of dGTP, we have analyzed our preparations for such a possible contaminant by three distinct procedures. Thin-layer chromatography was performed by ascending chromatography in a saturated $(NH_4)_2SO_4$–1 M NaOAc–2-propanol (40:9:1) solvent system. Gel filtration was performed on Sephadex G-25 in 5 mM NH_4HCO_3, pH 8, according to the protocol of Masamune & Richardson (1977). Sedimentation equilibrium was carried out in a double-sector synthetic boundary cell at an average speed of 46 000 rpm in a Model E analytical ultracentrifuge. None of these analyses revealed even low levels of oligo(ribonucleotides) (≤1%) contaminating our dGTP preparations. Furthermore, no detectable incorporation of dNTPs was observed when either oligo(dT)$_{12-18}$ was excluded from the reaction mixtures or oligo(dG)$_{12-18}$ was substituted for oligo(dT)$_{12-18}$ as primer (Collett et al., 1977).

Electrophoretic Analysis of cDNA. Electrophoretic sizing of DNA product was accomplished by using 3.5% polyacrylamide gels made in 98% formamide (denaturing gels), as has been previously described (Collett & Faras, 1977; Duesberg & Vogt, 1973; Maniatis et al., 1975). Dried samples were resuspended in 25 μL of deionized formamide. Deionization of the formamide was accomplished by the addition of 5 g of Bio-Rad AG-510-X8(D) mixed-bed resin to 100 mL of formamide and stirring at room temperature for 1 h. The resin was removed by filtration through Whatman GF/C filters. The DNA samples were then heated at 60 °C for 10 min and quenched on ice. Five microliters of 20 mM (Na_2H–NaH_2)PO_4 buffer, pH 7.5, containing 0.03% (w/v) bromphenol blue and 0.03% (w/v) xylene cyanol–FF was added. After the samples were layered onto 6 × 100 mm gels in Lucite tubes, electrophoresis was carried out in 20 mM (Na_2H–NaH_2)PO_4, pH 7.5, under a constant voltage of 140 V for 2.75 h or until the XC–FF tracking dye had migrated 50 mm. Gels were frozen on dry ice, sliced into 1 mm sections, and counted directly in a Beckman Biogamma II counter. Sizing of cDNA products has been independently confirmed by electroelution of product DNA from these gels, followed by electrophoresis on alkaline agarose gels as previously described (Clayman et al., 1979).

Results

Effect of Cation Concentration on the Synthesis of Full-Length DNA Transcripts in Reconstructed Reactions. In an attempt to delineate the reaction conditions required to facilitate uninterrupted elongation of DNA, we have employed AMV 35S RNA·oligo(dT)$_{12-18}$ template·primer complexes in enzymatic reactions containing purified reverse transcriptase under a variety of reaction conditions, followed by analysis of the resultant DNA product by electrophoresis in polyacrylamide gels under denaturing conditions in 98% formamide. AMV RNA was the template of choice in these experiments because of its length (~7500 nucleotides) and availability in our laboratory. When this type of analysis was

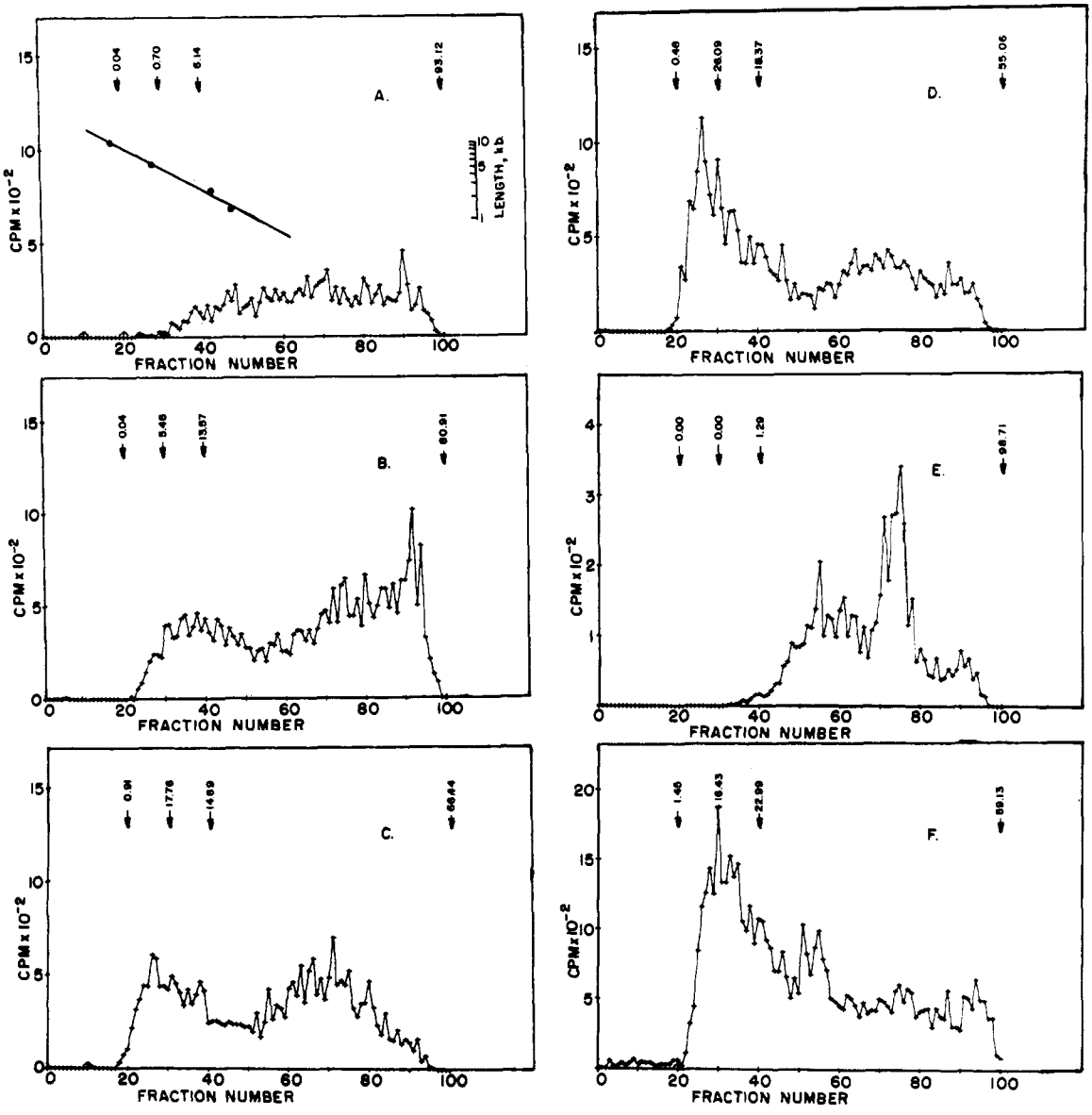

FIGURE 1: Effects of varying the monovalent cation concentration on DNA elongation during reverse transcription. Enzymatic synthesis of DNA from 35S RNA·oligo(dT)$_{12-18}$ template·primer complexes was performed as described under Materials and Methods except that the NaCl concentration was varied as indicated below. Following the incubation period, the DNA product was purified and analyzed on 3.5% polyacrylamide–98% formamide gels as described under Materials and Methods. The numbers above the arrows in all of the panels indicate the percentages of total radioactivity present between the arrows. For example, the first arrow indicates the percent of radioactivity between the ordinate and fraction 20, whereas the second arrow (from left to right) indicates the percent of radioactivity between fractions 20 and 30, etc. As indicated by the nucleotide-length standard curve (obtained with restriction endonuclease fragments of bacteriophage λ and P22 and form 3 SV40 DNA) in Figure 1A, DNA transcripts representing the entire AMV genome (i.e., 7500 nucleotides in length) migrate at approximately fraction 25 in this gel system. (Panel A) Enzymatic synthesis of DNA performed in the absence of NaCl; (panel B) enzymatic synthesis of DNA performed in the presence of 56 mM NaCl; (panel C) 111 mM NaCl; (panel D) 148 mM NaCl; (panel E) 214 mM NaCl; and (panel F) 148 mM KCl.

performed to determine optimum conditions of all the components employed in enzymatic reactions containing reverse transcriptase, monovalent cation concentration exhibited the most appreciable effect on the size distribution of DNA transcripts synthesized in reconstructed reactions. As seen in Figure 1, increasing the NaCl concentration in the reaction mixture from zero to near physiological (i.e., 148 mM) not only dramatically affects the size distribution of the DNA transcripts but also facilitates the synthesis of long cDNA transcripts as well (Figure 1A–D). In fact, under these particular conditions of DNA synthesis, the bulk of the DNA transcripts synthesized after 1 h of incubation at 37 °C migrate between 5000 and 7500 nucleotides in length (Figure 1D). Since the size of the AMV genome is ~7500 nucleotides in length, these data indicate that full-length DNA is being synthesized under these conditions. Increasing the NaCl concentration to 214 mM significantly inhibits synthesis of the longer DNA transcripts (Figure 1E). Concentrations of monovalent cation optimal for elongation of DNA during reverse transcription were partially inhibitory (~20–30%) to the total incorporation of deoxynucleoside triphosphates into the DNA product. Substituting the Na^+ with K^+ at physiological concentrations also enhanced the synthesis of longer DNA transcripts, including those that were genome-length in size (Figure 1F).

Because of the appreciable effect of a specific concentration of monovalent cation on the elongation of DNA transcripts during reverse transcription in vitro, we proceeded to optimize the reaction conditions for other components in the presence of optimal concentrations of monovalent cation in an effort

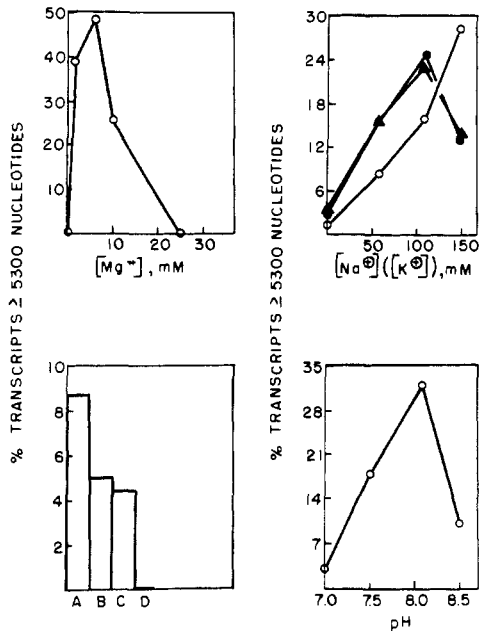

FIGURE 2: Effect of varying the reaction conditions on elongation during reverse transcription in reconstructed reactions. Within the context of the basic conditions outlined under Materials and Methods, all components of the reaction were investigated for their effect on transcript length, with the products analyzed on denaturing acrylamide gels. Data are presented as the percentage of total DNA product having a length greater than 5300 nucleotides. (Upper left) Magnesium concentration (1.3, 6.0, 10.0, and 24.6 mM). (Upper right) Monovalent cation concentration at different temperatures: (closed circles) Na^+, 37 °C; (closed triangles) K^+, 41 °C; (open circles) Na^+, 41 °C. Monovalent cation concentrations in the reaction were 0, 56, 111, and 148 mM. Increasing the monovalent cation concentration to 214 mM has the effect seen in Figure 1E. (Lower left) Effect of various concentrations of deoxynucleoside triphosphates using optimum concentrations of monovalent (148 mM NaCl) and divalent (6 mM $MgCl_2$) cations: (A) all dNTPs at 75 μM; (B) all dNTPs at 1 mM; (C) all dNTPs at 75 μM in the presence of 1 mM rNTPs; (D) dGTP, dTTP, and dATP at 75 μM and dCTP at 7.5 μM. (Lower right) Effects of varying the pH of the Tris buffer on the transcript length (pH 7.0, 7.5, 8.1, and 8.5) at 75 μM dNTPs, 148 mM NaCl, 6 mM Mg^{2+}, and 41 °C.

to determine the precise enzymatic requirements for efficient full-length DNA transcription. The effect of varying the concentration of divalent cation on the synthesis of genome-length DNA from 35S RNA·oligo(dT)$_{12-18}$ template·primer complexes is shown in Figure 2. As was the case for monovalent cation, divalent cation also exhibits a relatively specific concentration range in which full-length DNA transcription is optimal. In these experiments, 6 mM Mg^{2+} appeared to exhibit the most profound effect, with higher concentrations of Mg^{2+} resulting in high yields of incomplete transcripts. Mn^{2+} could be substituted for Mg^{2+} in these reactions; however, the yields of full-length DNA were not as high as those observed for Mg^{2+} (data not shown). In contrast to monovalent cation, no inhibition of incorporation of dNTPs into DNA product was observed with optimum concentrations of divalent cation.

Effect of Deoxynucleoside Triphosphate Concentration on the Synthesis of Full-Length DNA. Previous analysis of enzymatic synthesis of DNA in reconstructed reactions indicated that increasing the concentration of deoxynucleoside triphosphates significantly affected the size of the DNA transcripts synthesized in these reactions, although little, if any, full-length DNA could be detected (Collett & Faras, 1977). In an effort to determine if the dNTP concentration affected DNA synthesis in reconstructed reactions containing optimal concentrations of monovalent and divalent cations, we analyzed the size of the DNA product synthesized in the presence of varying concentrations of nucleotide precursor in 98% formamide–3.5% polyacrylamide gels (Figure 2). Under rate-limiting concentrations of dNTPs (7.5 μM radiolabeled dCTP and 75 μM unlabeled dGTP, dATP, and dTTP), the bulk of the DNA product remained small in size with none of the DNA transcripts exhibiting lengths of 3000 nucleotides or more even when enzymatic synthesis was performed in the presence of 148 mM salt. Increasing the rate-limiting nucleotide (dCTP) to a concentration equal to the remaining three deoxynucleoside triphosphates (75 μM) exhibited an appreciable effect on the length of the DNA transcripts. Moreover, no significant additional differences were observed in the efficiency of full-length DNA transcription when the dNTP concentration was further increased to 1×10^{-3} M. Finally, since in vitro reverse transcription studies have indicated that 1×10^{-3} M ribonucleoside triphosphates could facilitate the synthesis of genome-length DNA that was infectious in nature even in the presence of low concentrations of dNTPs (Clayman et al., 1979), we have also tested the effect of rNTPs on DNA synthesis in reconstructed reactions. In contrast to previous reports describing optimal conditions for enzymatic synthesis of DNA in reactions containing either detergent-disrupted RSV (Clayman et al., 1979) or partially purified reverse transcriptase (Kacian & Myers, 1976), no appreciable effect of rNTPs on elongation was observed in the reconstructed reactions containing rigorously purified (i.e., lacking all residual RNase) reverse transcriptase. Since the effect of high rNTPs on uninterrupted DNA synthesis presumably reflects an inhibition of virion-associated nucleases (Clayman et al., 1979; Kacian & Myers, 1976), no significant effect on the length of the DNA transcripts would be expected in reconstructed reactions which lack both detectable nuclease activity and observable template degradation during enzymatic synthesis (Collett & Faras, 1977).

Effect of Temperature and pH on the Synthesis of Genome-Length DNA Transcripts. The remainder of the reaction conditions that were tested for a possible effect on the synthesis of full-length DNA from 35S RNA·oligo(dT)$_{12-18}$ template·primer complexes included temperature, pH, phosphate ion concentration, and buffer system. In the temperature experiments, monovalent cation concentration was varied at three different temperatures (37, 41, and 46 °C) and the resultant DNA transcripts were sized on 98% formamide–3.5% polyacrylamide gels (Figure 2). Irrespective of the temperature, the optimum salt concentration for maximum genome-length DNA synthesis in these enzymatic reactions remained at 148 mM. Although a slight decrease in the yield of genome-length DNA was observed at 46 °C, no dramatic differences in the synthesis of full-length DNA were observed at 37 or 41 °C, indicating that either temperature was satisfactory for maximum elongation in the presence of physiological salt concentration (Figure 2).

Since temperature has an effect on the pH of the buffer system employed in these reconstructed reactions, the pH of the buffer (Tris-HCl) was monitored at the temperature employed for enzymatic synthesis. Several pH values were tested to determine the effect, if any, of hydrogen ion concentration on the synthesis of full-length DNA in these reactions. Employing the optimum conditions delineated for maximum elongation of DNA transcripts (148 mM monovalent cation, 6 mM Mg^{2+}, 75 μM dNTPs, 41 °C), we have analyzed the effect of pH on the length of the DNA synthesized in reconstructed reactions. From the data presented in Figure 2, it

Table I: Protection of Viral RNA by DNA Transcripts Synthesized under Optimal Conditions for Full-Length DNA Transcription[a]

DNA/RNA ratio	% RNase resistance	DNA/RNA ratio	% RNase resistance
1:1	62	4:1	86
2:1	74	8:1	92

[a] Total DNA product prepared under conditions of optimal genome-length DNA synthesis employing 35S RNA·oligo(dT)$_{12-18}$ template-primer complexes (i.e., 148 mM NaCl, 6 mM $MgCl_2$, 75 μM dNTPs, 41 °C) was hybridized to ^{125}I-labeled viral RNA at the ratios of DNA to RNA indicated as previously described (Collett & Faras, 1977). Subsequent to hybridization, the samples were treated with 40 μg/mL pancreatic RNase in 2× SSC (0.3 M NaCl and 0.03 M sodium citrate) for 30 min at 37 °C. Data are presented as an average of duplicate samples.

is apparent that the greatest yields of full-length DNA were obtained at a $pH_{25°C}$ of 8.1, the pH value that also exhibited a maximum effect on the incorporation of deoxynucleoside triphosphates into acid-insoluble material (Faras et al., 1972).

Although other buffer systems were tested for their effect on full-length DNA synthesis (e.g., PO_4^{2-} and cacodylate), none appeared to be better than the Tris-HCl buffering system employed in the aforementioned studies. Furthermore, when phosphate ion was tested at or near physiological concentrations, an inhibitory effect on the elongation reaction was observed since the DNA transcripts were appreciably smaller in size than those observed in enzymatic reactions lacking phosphate (E. Retzel and A. Faras, unpublished experiments).

Genetic Complexity of Genome-Length DNA Transcripts Synthesized under Optimum Reaction Conditions. In order to demonstrate that the genome-length DNA transcripts synthesized under optimum reaction conditions from 35S RNA·oligo(dT)$_{12-18}$ template-primer complexes contain most, if not all, of the genetic sequences present in the viral genome, we have analyzed the genetic complexity of the DNA product by hybridization–protection experiments (Collett & Faras, 1977). In this analysis, the genetic complexity of the DNA was determined by the extent to which unselected DNA transcripts protected radiolabeled genomic RNA from RNase hydrolysis (Table I). Since these DNA transcripts protected the majority of the viral genome (92%) from RNase hydrolysis at low DNA/RNA ratios (i.e., 8:1), the bulk of the viral genomic sequences must have been transcribed into DNA under these reaction conditions.

Discussion

In this communication, we present data indicating that cDNA transcripts representing the entire length of high molecular weight RNA molecules can be synthesized in reconstructed reactions containing purified viral RNA and reverse transcriptase if the specific conditions of enzymatic synthesis are optimized for elongation of DNA transcripts. The most dramatic effect on the elongation of DNA in these reactions was observed with a relatively high monovalent cation concentration, although clearly other parameters (divalent cation, dNTP, pH, temperature, etc.) also exhibited an effect on this reaction. To our surprise, the concentration of monovalent cation required to facilitate maximum full-length DNA transcription was near the physiological concentration present within the cell. The reason for this requirement of near-physiological salt concentration in reconstructed reactions is not known; however, there are several possibilities worthy of consideration. First, high monovalent salt concentration could conceivably inhibit residual contaminating RNase activity present in the reverse transcriptase preparations (Collett & Faras, 1975a,b). However, we routinely subject our AMV reverse transcriptase preparations to further purification to remove such activities (see Materials and Methods). Moreover, sensitive procedures to detect as little as one nick per RNA molecule are employed to rigorously assay the purified enzyme for the presence of RNase. Second, since high monovalent cation concentration appears to reduce the Mg^{2+}-induced degradation of viral RNA reported in the endogenous system (Rothenberg & Baltimore, 1977; E. Retzel, unpublished experiments), we have assayed this effect on template degradation in our reconstructed reactions. No degradation was observed if our enzyme preparations employed in the reactions were pure and if Mg^{2+} was chelated prior to heat denaturation. Third, since reverse transcriptase aggregates in buffers lacking monovalent cation (Faras et al., 1972), it was conceivable that such an effect was responsible for incomplete transcription because of the low enzyme/RNA ratio. However, saturation curves of the enzyme in the presence and absence of 148 mM Na^+ were similar, indicating that although the reverse transcriptase may aggregate in the absence of salt, its availability for transcription of RNA was not affected (E. Retzel and A. Faras, unpublished experiments). Fourth, it was possible that the NaCl concentration affects the secondary structure of the RNA template and/or its affinity to bind to the reverse transcriptase. However, neither of these explanations appear very convincing, since high salt tends to stabilize rather than destabilize the secondary structure of the RNA (Duesberg, 1968; Stoltzfus & Snyder, 1975), and the predicted effect of ligand–nucleic acid binding would be one of dissociation rather than association (Record et al., 1976). The presence of high salt in the reaction mixtures may, on the other hand, reduce the nonspecific binding of the enzyme to the template (E. Retzel and A. Faras, unpublished experiments; Hizi et al., 1977). Finally, the NaCl concentration may affect an unwinding-like activity that we have recently identified associated with preparations of AMV reverse transcriptase (Collett et al., 1978a). Under conventional conditions (low monovalent cation concentration) of enzymatic synthesis (Collett & Faras, 1977), this unwinding-like activity disrupts the resultant RNA–DNA hybrids, rendering the DNA sensitive to the single-strand-specific nuclease S1 (Collett et al., 1978a,b). It is conceivable that such disruption aborts elongation directly, possibly by removing the 3′ terminus of the DNA transcripts from the RNA template during transcription. Since at least a portion of the DNA product synthesized in reconstructed reactions exhibits resistance to exonuclease I hydrolysis (Collett et al., 1978a,b), it is possible that re-formation of a hybrid structure capable of serving as a template-primer for further elongation is prohibited by DNA transcripts containing hairpin structures at their 3′ termini (Collett & Faras, 1978). Although purely speculative, this explanation is supported to some extent by the observation that the unwinding activity is inhibited by physiological concentrations of salt, at least under the conditions of enzymatic synthesis employed in the studies reported in this communication. Moreover, a substantial reduction in hairpin DNA synthesis is observed under these reaction conditions. Nevertheless, further studies will be required before this explanation can be confirmed or refuted.

Irrespective of the precise mechanism of the effect of physiological monovalent cation concentration on reverse transcription, the data presented in this communication should provide useful information to those who require a reproducible set of enzymatic conditions to facilitate the uninterrupted transcription of high molecular weight RNA molecules.

Whether these requirements include the use of complete cDNA copies of RNA for probes in hybridization experiments or for cloning in bacteria, the conditions described herein alleviate any previous limitations due to the length of the RNA template employed.

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Nonuniform Backbone Conformation of Deoxyribonucleic Acid Indicated by Phosphorus-31 Nuclear Magnetic Resonance Chemical Shift Anisotropy†

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ABSTRACT: ^{31}P nuclear magnetic resonance of highly oriented DNA fibers has been observed for three different conformations, namely, the A, B, and C forms of DNA. At a parallel orientation of the fiber axis with respect to the magnetic field, DNA fibers in both the A and B forms exhibit a single, abnormally broad resonance; in contrast, fibers in the C form show almost the full span of the chemical shift anisotropy (170 ppm). The spectra of the fibers oriented perpendicular indicate that the DNA molecules undergo a considerable rotational motion about the helical axis, with a rate of $>2 \times 10^3$ s^{-1} for the B-form DNA. Theoretical considerations indicate that the ^{31}P chemical shift data for the B-form DNA fibers are consistent with the atomic coordinates of the phosphodiester group proposed by Langridge et al. [Langridge, R., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F., & Hamilton, L. D. (1960) *J. Mol. Biol. 2*, 19-37] but not with the corresponding coordinates proposed by Arnott and Hukins [Arnott, S., & Hukins, D. W. L. (1972) *Biochem. Biophys. Res. Commun. 47*, 1504-1509], and also lead to the conclusion that the phosphodiester orientation must vary significantly along the DNA molecule. The latter result suggests that DNA has significant variations in its backbone conformation along the molecule.

DNA[1] in aqueous solution has generally been assumed to adopt the same conformation as is present in DNA fibers equilibrated at high relative humidities, namely, the B form of DNA (Langridge et al., 1960). Recently, several studies have proposed alternative secondary structures for DNA in solution based on a variety of techniques [for references, see

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[1] Abbreviations used: NMR, nuclear magnetic resonance; CSA, chemical shift anisotropy; PAS, principal axis system; DNA, deoxyribonucleic acid.