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The Synthesis and Properties of the Complete Complementary DNA Transcript of Ovalbumin mRNA†

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ABSTRACT: The synthesis of a complementary DNA copy (cDNA) of hen ovalbumin mRNA using AMV RNA-directed DNA polymerase was studied under different conditions of salt, deoxyribonucleotide concentrations, temperature, and time. It was observed that in the absence of monovalent cation at 46°C a complete transcript of ovalbumin mRNA could be effected by the enzyme. The minimum deoxyribonucleotide requirement for complete synthesis was 35 μM for dATP, dGTP, and dCTP and 200 μM dTTP. By a number of different experimental criteria which included sedimentation on alkaline sucrose gradients and electrophoresis in polyacrylamide gels containing 98% formamide, direct electron microscope visualization, and protection of ovalbumin [^{125}I]mRNA from nuclease digestion it could be demonstrated that a considerable fraction of a complete mRNA transcript was indeed synthesized. The cDNA/ovalbumin mRNA hybrid had a T_m on hydroxylapatite of 92°C, indicating the synthesis of a RNA transcript with a high fidelity. When such a complete ovalbumin [^{3}H]cDNA was synthesized with a specific activity of 10^8 cpm/μg and hybridized to an excess of chick DNA, the kinetics of hybridization indicated that the cDNA was comprised of a nonrepetitive sequence.

RNA-directed DNA polymerases (reverse transcriptase) isolated from avian myeloblastosis virus (AMV) and Rous sarcoma virus (RSV) have been used to synthesize cDNAs[1] that are complementary to eucaryotic cell messenger RNAs (Verma et al., 1972, 1974; Ross et al., 1972; Honjo et al., 1974; Thrall et al., 1974; Diggelman et al., 1973; Harris et al., 1973; Bishop et al., 1974). Such cDNAs have become widely employed as sensitive and specific hybridization probes in a variety of experiments designed to study gene frequency, the transcription of chromatin in vitro and HnRNA and mRNA metabolism (see review of Lewin, 1975; Rosen and O'Malley, 1975). It is generally observed that the average size of the cDNA product is smaller than that of the RNA template. For example with rabbit globin mRNA as template (10 S), cDNA products 5 S to 8 S have been described (Ross et al., 1972). Complementary DNA from MOPC 41 myeloma immunoglobin light chain mRNA (14 S) had a major component of about 5 S (Honjo et al., 1974; Diggelman et al., 1973). With ovalbumin mRNA, cDNA has been described which was heterogeneous in size with an average sedimentation coefficient of 5 S

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[1] Abbreviations used are: $cDNA_{ov}$, ovalbumin complementary DNA; AMV, avian myeloblastosis virus; NT, nucleotides; dNTP, deoxyribonucleoside triphosphate; EDTA, disodium ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxypiperazine-*N'*-2-ethanesulfonic acid; C_0t, the product of the total RNA or DNA concentration in moles of nucleotides per liter and the time in seconds.

to 7 S (Harris et al., 1973; Cox et al., 1974; Sullivan et al., 1973). It has been reported that the average size of the cDNA product can be increased by the presence of relatively high concentrations of dNTP's in the reaction mixture (Faust et al., 1973; Imaizumi et al., 1973). Efstratiadis et al. (1975) have also recently reported the synthesis of rabbit globin cDNA that contains a considerable proportion of cDNAs that represent complete transcripts of the globin mRNA template using 30–50 μM dNTP substrate concentrations. However, when the longer ovalbumin mRNA template was used (and also a higher dNTP concentration, 100 μM of each) much of the $cDNA_{ov}$ product formed was only about 900 nucleotides long (Efstratiadis et al., 1975). In most cases a cDNA product labeled to a very high specific activity is required for nucleic acid hybridization studies designed to measure unique sequence DNA transcript under conditions of DNA excess (Bishop, 1972). In such cases it would be desirable to synthesize the cDNA with low dNTP substrate concentrations. A cDNA labeled to a high specific activity is also required for in situ hybridization studies (Gall and Pardue, 1971).

In this paper we have used a relatively large mRNA, ovalbumin mRNA, as a model system to find suitable conditions whereby AMV reverse transcriptase is capable of copying the complete mRNA template to yield a cDNA product (long $cDNA_{ov}$) with a specific activity of 10^8 cpm/μg. Such cDNAs have a number of important potential uses.

Materials and Methods

Isolation of Ovalbumin mRNA. Purified ovalbumin mRNA was isolated as we have described previously (Rosen et al., 1975; Woo et al., 1975). Briefly, frozen hen oviducts were extracted twice at room temperature with 10 volumes of phenol saturated-sodium dodecyl sulfate-EDTA buffer (pH 8.0). The total nucleic acid extract was precipitated with ethanol, centrifuged, and redissolved in water. The total extract was then placed on an oligo(dT)-cellulose column. Affinity chromatography using dT-cellulose was performed at room temperature as described by Aviv and Leder (1972), with the omission of the 0.1 M KCl intermediate salt wash. The RNA bound to the oligo(dT)-cellulose was subjected to fractionation on the basis of size and secondary structure by gel filtration on a Sepharose 4B column (2.6 × 100 cm) as described previously (Woo et al., 1974). The fractions containing ovalbumin mRNA activity as assayed by the wheat germ translation system (Roberts and Patterson, 1973) were pooled and rechromatographed on a second oligo(dT)-cellulose column. The small amount of contaminating messenger RNAs and residual 18S rRNA still present in the bound RNA fraction were removed by preparative agarose gel electrophoresis using an acid-urea 2% agarose gel system (Woo et al., 1975). The purity of the ovalbumin mRNA has been previously determined by biological, physical, and chemical methods. In addition each preparation was routinely checked by analytical procedures such as acid-urea agarose gel electrophoresis, polyacrylamide gel electrophoresis in 98% formamide, and sucrose gradient centrifugation in 70% formamide. These procedures have been described in detail elsewhere (Rosen et al., 1975). Only ovalbumin mRNA of greater than 99% purity was used in these studies.

Iodination of RNA. Iodination of RNA was carried out by a modification of the procedure of Commerford (1971). The reaction mixture was composed of 0.1 M sodium acetate (pH 5.0), 5×10^{-5} M potassium iodide, 7.5×10^{-4} M thallium chloride, 0.5 mg/ml of RNA and 50 mCi/ml of carrier free $Na^{125}I$ in a total volume of 50 μl. After the reaction was allowed to proceed at 60°C for 15 min, 5 μl of 0.01 M tyrosine was added to remove the unreacted iodine. Ten microliters of 2.8 M sodium phosphate (pH 6.8) was added and the mixture was again incubated at 60°C for 15 min to destroy the unstable intermediates. The entire reaction mixture was cooled to room temperature and passed through a small Sephadex G-50 column (0.6 × 30 cm) in deionized water. Fractions in the void volume that contained radioactivity were pooled, and the labeled RNA was precipitated from ethanol at −20°C overnight after the addition of KCl to 0.5 M. The [^{125}I]RNA was then further purified by sedimentation through 0.3–1.0 M sucrose gradients containing 0.01 M sodium acetate (pH 5.0), 0.1 M NaCl, and 1 mM EDTA. Centrifugation was carried out for 16 hr at 35000 rpm as described previously (Rosen et al., 1974). Fractions of constant specific radioactivity were pooled. RNA labeled in this manner yielded specific radioactivities of $1–2 \times 10^7$ cpm/μg. The purified ^{125}I-labeled ovalbumin mRNA comigrated with unlabeled ovalbumin mRNA on acid-urea agarose gels as demonstrated by autoradiography.

Purification of Reverse Transcriptase. Highly purified avian myeloblastosis virus reverse transcriptase was generously supplied by Dr. J. W. Beard (Life Sciences, Inc., St. Petersburg, Fla.). The purification procedure was a modification of that of Kacian and Spiegelman (1973) and will be described in detail elsewhere (Houts and Beard, personal communication). In summary, purified virions of AMV were lysed with nonionic detergent and 0.5 M KCl. The clarified crude extract was passed through a DEAE column. Reverse transcriptase was batch-eluted from the DEAE column and adsorbed to a phosphocellulose column. The enzyme fractions were eluted from the phosphocellulose column with a linear phosphate gradient. Active fractions of reverse transcriptase were pooled, diluted, and rechromatographed on a second phosphocellulose column. The final pool of reverse transcriptase fraction was concentrated by dialysis against 50% glycerol, 0.2 M potassium phosphate (pH 7.2), 2 mM dithiothreitol, and 0.2% Triton X-100 and stored at −20°C. We have obtained long $cDNA_{ov}$ from five different batches of enzyme, obtaining the same results in all cases.

Centrifugation of cDNA in Alkaline Sucrose Gradients. Labeled cDNA after gel filtration and alkaline hydrolysis was precipitated with ethanol, redissolved in 0.1 ml of a mixture of 0.1 M NaOH, 0.9 M NaCl, and 5 mM EDTA, and layered onto a 8–18% linear sucrose gradient in the same solution. The gradient was centrifuged for 24 hr at 38000 rpm, 5°C, in a Beckman SW40 rotor. Fractions were neutralized and counted in Aquasol. Sheared *Escherichia coli* DNA (3.8 S and 5.08 S) as well as the linear (16.1 S) and the circular form (18.4 S) of ϕX174 DNA were used as markers. The s values ($s_{20,w}^{pH\ 13}$) of the *E. coli* DNAs were obtained by analytical sedimentation measurements in a Spinco Model E ultracentrifuge using the alkaline buffer described above without sucrose. The s values of the open and closed forms of ϕX174 DNA in alkali have been previously reported by Studier (1965).

Purification of Chick DNA. Total chick liver DNA was prepared by a modification of the procedure of Marmur (1961) as described previously (Rosen et al., 1973). The DNA preparations used were essentially free of RNA and

protein. The final DNA solution was adjusted to approximately 1 mg/ml in 0.01 *M* Tris (pH 7.6) containing 0.2 *M* NaCl and sheared twice in a French Press to yield DNA fragments which were ~400 base pairs in length. Proteinase K was then added to 50 μg/ml and dodecyl sulfate to 0.5% and the solution was incubated for 1 hr at 37°C to remove any possible trace contamination of nuclease. The solution was then vigorously shaken for 5 min with an equal volume of phenol–dodecyl sulfate buffer (pH 8.0) identical with that used for total nucleic acid extraction. The mixture was centrifuged and the aqueous phase removed and reextracted with chloroform (Marmur, 1961) two additional times. The DNA in the aqueous phase was then precipitated with ethanol, redissolved to about 8 mg/ml in 0.1 m*M* EDTA, heat denatured, and stored as a stock solution. *E. coli* DNA was prepared essentially as described above. The short DNA fragments (3.8 S) were prepared by shearing twice in a French Press as described above except 0.5% dodecyl sulfate was also present in the shearing buffer.

DNA and mRNA Excess Hybridization to Ovalbumin cDNA. DNA and RNA excess hybridization experiments with the $cDNA_{ov}$ were carried out in tapered reaction vials (Regis Chemical Company, Ill.). During the incubation, the vials were submerged to prevent condensation. The hybridization reaction was performed in a final volume of 50 μl containing: 0.6 *M* NaCl, 0.01 *M* Tris-HCl, 0.001 *M* Hepes (pH 7.0 at 25°C), and 0.002 *M* EDTA. Two to four samples per experiment were assayed with no added ovalbumin mRNA for determination of the total radioactivity that was Cl_3CCOOH precipitable prior to treatment with S_1 nuclease vs. the total radioactivity remaining after S_1 nuclease treatment. Generally less than 2% of the total radioactivity was resistant to S_1 nuclease. Samples were initially placed in a boiling water bath for 30 sec and then incubated at 68°C for time intervals ranging from 0.1 to 64 hr, depending on the cDNA preparation being tested. For DNA excess hybridization experiments the initial stock solution of chick DNA was denatured by heating to 100°C for 10 min in 0.05 *M* EDTA–0.01 *M* NaCl–0.01 *M* Tris-HCl (pH 8.0). After hybridization the vials were quickly frozen in liquid nitrogen.

To assay the extent of hybridization 0.2 ml of a solution containing 0.4 *M* sodium acetate (pH 4.5), 0.8 *M* NaCl, 5 m*M* $ZnCl_2$, and 0.15 ml of the pooled S_1 nuclease peak from the final DEAE-cellulose column (Harris et al., 1975) was added to each vial. The vials were incubated for 2 hr at 30°C. The S_1 nuclease resistant hybrids were precipitated with cold 20% Cl_3CCOOH, collected on Millipore filters, and dried. The dried filters were then dissolved in 3 ml of "Cellusolve" (ethylene glycol monomethyl ether) by shaking for 2 hr at room temperature and counted in 10 ml of Aquasol.

Both the $C_0t_{1/2}$ values and the hybridization curves were determined using a computer program designed to fit the curve to the date defined by eq 1 for cDNA hybridization under conditions of an mRNA excess, or eq 2 for cDNA hybridization under conditions of vast DNA excess. d/D_0 = fraction of hybrid formed, P = final extent of hybridization, C_0t = mol sec $l.^{-1}$ of nucleotides DNA or RNA. Details of the computer program will be described in detail elsewhere (J. J. Monahan, S. E. Harris, and B. W. O'Malley, manuscript in preparation).

$$d/D_0 = P[1 - \exp((-\ln 2)C_0t)/C_0t_{1/2}] \quad (1)$$

$$d/D_0 = P[1 - (C_0t/C_0t_{1/2} + 1)^{-1}] \quad (2)$$

Preparative Scale Synthesis of Ovalbumin cDNA. The procedure for synthesis of complete ovalbumin cDNA labeled with ^3H to a specific activity of 10^8 cpm/μg was as follows: 2 mCi each of [^{3}H]dGTP, [^{3}H]dATP, and [^{3}H]dCTP were lyophilized to dryness and were resuspended in 1 ml of a solution containing 50 m*M* Tris-HCl (pH 8.3), 20 m*M* dithiothreitol, 6 m*M* $MgCl_2$, 200 μ*M* dTTP, 2.5 μg/ml of oligo(dT_{18-20}), 36 μg/ml of actinomycin D, 1% ethanol, and 25 μg/ml of ovalbumin mRNA. The actinomycin D was made up as a stock solution of 360 μg/ml in 10% ethanol. The final concentrations of the [^{3}H]dGTP, [^{3}H]dATP, and [^{3}H]dCTP were adjusted to 35 μ*M*. The mixture was left on ice for 5 min. Then reverse transcriptase enzyme was quickly added to a final concentration of 60 units/ml. The solution was quickly vortexed and incubated for 5 min at 46°C. The reaction mixture was then made 0.5% with sodium sarcosyl, 10 m*M* EDTA, and 100 μg/ml with sheared *E. coli* DNA. The entire sample was placed on a Sephadex G-50 column equilibrated with 0.1 *M* NaCl–0.01 *M* Tris (pH 7.6). The excluded fraction was collected and precipitated with 2 volumes of ethanol in 0.2 *M* sodium acetate (pH 5.5). The precipitate was dissolved in 0.3 ml of 0.01 *M* EDTA–0.1 *M* NaOH and heated to 60°C for 30 min. After readjusting the solution to pH 5.5 the cDNA (now free of RNA) was then precipitated with ethanol.

Analytical Scale Synthesis of Ovalbumin cDNA. For experiments in which the effects of different concentrations of salt, deoxyribonucleotide concentration, temperature, and time on cDNA synthesis were tested, 50 Ci/ml of [^{32}P[dGTP was used to monitor the synthesis of cDNA. The final volume of the reaction mixture was 0.1 ml. After completion of cDNA synthesis, the reaction mixture was made 0.5% with sodium sarcosyl, 10 m*M* EDTA, and 0.1 *M* NaOH. Marker DNAs were added to the solution which was then layered on an alkaline sucrose gradient and centrifuged as described above. The distribution of cDNA in the gradient was determined by observing the Cl_3CCOOH precipitable radioactivity in the gradient.

Electron Microscopy. Samples for electron microscopy were prepared by the formamide modification of the Kleinschmidt technique of Davis et al. (1971). The grids were rotary shadowed with platinum–palladium (80:20) and examined in a Philips 300 electron microscope. Lengths of single-strand DNA were determined relative to an internal standard of ϕx 174 DNA.

Results

Synthesis of a Complete cDNA Copy of Ovalbumin mRNA. The optimum experimental conditions for the synthesis of cDNAs transcribed from either synthetic homo- or heteropolymers by reverse transcriptase isolated from a number of different sources have now been examined in some detail (Wu and Cetta, 1975; Smith and Gallo, 1974; Tamblyn and Well, 1975; Baltimore and Smoler, 1971; Hurwitz and Leis, 1972; Temin and Baltimore, 1972). The absolute requirement of a primer for transcription has been well demonstrated (Temin and Baltimore, 1972). This need for both template and primer, coupled with the numerous effects of monovalent and divalent cations on their secondary structure, gives rise to a diverse range of optimum conditions depending on the nature of the template used for the synthesis of the cDNA (Temin and Baltimore, 1972; Wu and Cetta, 1975; Marcus and Modak, 1975). There is as yet only limited data available for the optimum experimental

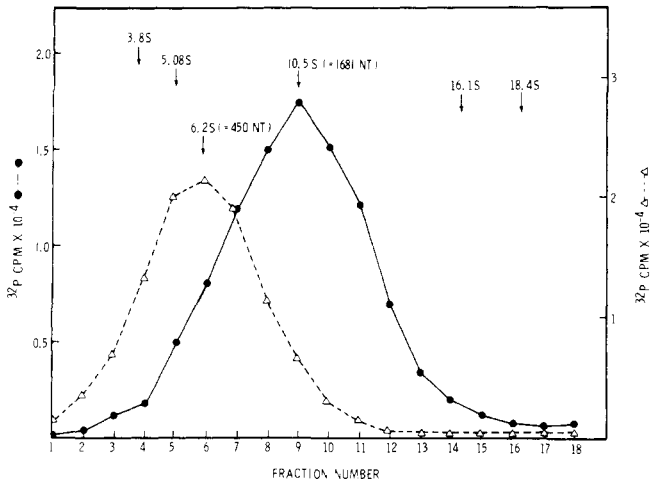

FIGURE 1: Alkaline sucrose gradient of [^{32}P]cDNA$_{ov}$ synthesized in (△- - -△) 50 m*M* Tris-HCl (pH 8.3), 20 m*M* dithiothreitol, 6 m*M* $MgCl_2$, 96 m*M* KCl, 200 μM dTTP, 35 μM each of dGTP, dCTP, dATP, 5 μg/ml of oligo (dT_{18-20}), 36 μg/ml of actinomycin D, 1% ethanol, 25 μg/ml of mRNA, 50 μCi/ml of [^{32}P]dGTP, and 60 units/ml of reverse transcriptase. Synthesis was for 1 hr at 37°C. (●—●) Alkaline sucrose gradient of "long" [^{32}P]cDNA$_0$ synthesized in the buffer described above with no KCl present. Synthesis was for 5 min at 46°C. The samples were centrifuged in a SW40 rotor at 5°C for 24 hr at 38000 rpm.

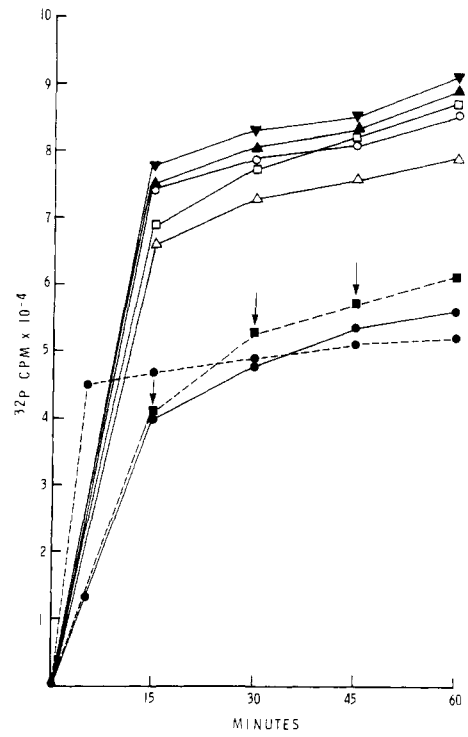

FIGURE 2: Effects of some reagents upon the synthesis of cDNA$_{ov}$. Buffer conditions were the same as those for the short cDNA$_{ov}$ of Figure 1 (●—●). Conditions were then changed to a final concentration of 5% glycerol (▲—▲), to a final concentration of 10% glycerol (▼—▼), to a final concentration of 0.01% Triton X-100 (○—○), to an oligo(dT) final concentration of 0.5 μg/ml (□—□), or to a final concentration of 10% Me_2SO (△—△). A further 10 units/ml of enzyme was added at 15, 30, and 34 min (■- - -■). Synthesis was also carried out as described above for short cDNA$_{ov}$ in Figure 1 except that the temperature was 46°C (●- - -●).

conditions necessary to synthesize cDNAs from natural mRNA templates. This has been due in part to the limited availability of purified mRNAs. Because large amounts of purified ovalbumin mRNA have now become available to us (O'Malley and Means, 1974), we have chosen this mRNA as our model template to determine the conditions necessary for the complete in vitro synthesis of cDNA$_{ov}$ labeled to a high specific activity using AMV reverse transcriptase.

In our initial studies and those of other laboratories, predominately short ovalbumin cDNAs were obtained (Sullivan et al., 1973; Harris et al., 1973; Cox et al., 1974; McKnight and Schimke, 1974). Figure 1 shows a typical profile of ovalbumin cDNA on an 8–18% alkaline sucrose gradient. The reaction was carried out under standard conditions generally used for cDNA synthesis using 96 m*M* KCl, 200 μM dTTP, and 35 μM each of dGTP, dCTP, and dATP as described under Materials and Methods. The total incubation time was 1 hr at 37°C. The cDNA product sedimented at 6.2 S corresponding to a chain length of approximately 450 nucleotides.

A number of parameters were then altered in the protocol for synthesis of cDNA to try to increase the size of the cDNA. It was important to establish if the small size of the cDNA was due to a failure of the reverse transcriptase to move along the whole length of the mRNA or if the reduction in size of already completed synthesized cDNAs was occurring as a result of DNase activity associated with the enzyme preparation. In order to resolve this question the following experiments were performed. First, no increase in size of the cDNA was seen when short incubation times (~5 min) were utilized. Second, there was no further reduction in size of the cDNA when a purified cDNA prepared as described above was reincubated with fresh enzyme under the above buffer conditions. These observations (together with hybridization data to be described below) suggested that the enzyme somehow was unable to transcribe the complete mRNA template and termination occurred after polymerization of only 450 nucleotides. Since the enzyme in this system had an absolute requirement for oligo(dT) primer (Harris et al., 1973), it seems reasonable to suggest that these short cDNAs probably only represent sequences complementary to the 3′-terminal end of ovalbumin mRNA.

A number of conditions were examined in order to facilitate a more extensive movement of the enzyme along the mRNA. It was observed, for example, that addition of 5 or 10% glycerol, 10% Me_2SO, or 0.01% Triton X-100 each gave a twofold increase in the yield of cDNA made but did not result in a larger cDNA product. Reducing the oligo(dT) primer concentration to 0.5 μg/ml had a similar effect. Figure 2 shows the extent of cDNA synthesis under the above conditions. We should point out that in all cases there was some glycerol (1.25%), and Triton X-100 (0.005%) in the reaction mixture used for cDNA synthesis since the enzyme itself was stored in a buffer containing glycerol and Triton X-100. The fact that the enzyme was capable of working in 10% Me_2SO was somewhat surprising to us. However, in all of the above cases the cDNA$_{ov}$ products obtained were short and similar to that of the short cDNA$_{ov}$ shown in Figure 1. Serial addition of further enzyme (10 units/ml) at 15, 30, and 45 min after the start of synthesis (Figure 2) also gave short cDNA$_{ov}$ similar to that shown in Figure 1. We speculate that this was due to degradation of the mRNA in the DNA–RNA hybrid formed during cDNA synthesis by an RNase H activity associated with the reverse transcriptase enzyme (Grandgenett et al., 1973; Watson et al., 1973).

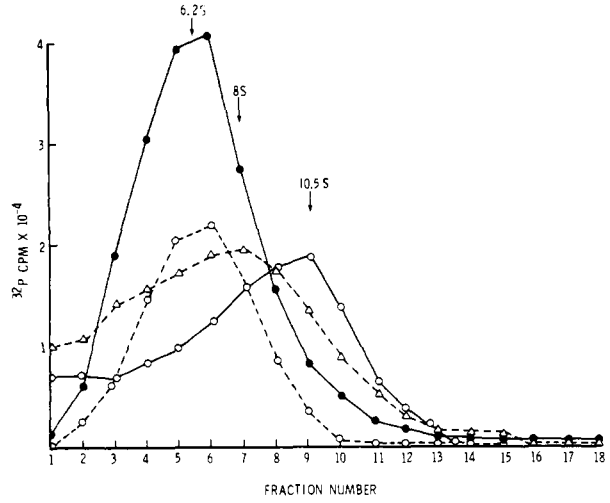

FIGURE 3: Effect of KCl concentration upon the length of $cDNA_{ov}$ synthesized. Buffer and centrifugation conditions were the same as for those for Figure 1. (O- - - -O) 0.1 *M* KCl; (●—●) 0.15 *M* KCl; (Δ- - - -Δ) 0.05 *M* KCl; (O—O) no KCl. Synthesis was for 1 hr at 37°C.

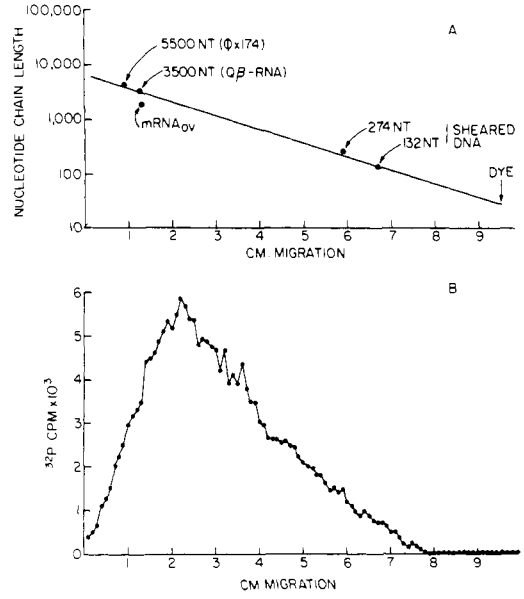

FIGURE 4: Polyacrylamide gel electrophoresis in 98% formamide of $cDNA_{ov}$. Long [^{32}P]$cDNA_{ov}$ synthesized as described in Figure 1 was analyzed on a 4% polyacrylamide gel. The positions of calibration markers ϕX174 (open) DNA, phage Qβ RNA, ovalbumin mRNA, and 5.08S and 3.8S *E. coli* DNAs are shown in A. The calibration markers were stained with "Stains All" (Dahlberg et al., 1969). The position of the long cDNA shown in B was determined by slicing the gel (1-mm slices) and counting. Electrophoresis was for 90 min at 5 mA/gel.

However, certain conditions were able to substantially alter the size of the cDNA product. The concentration of KCl used during the cDNA synthesis had a dramatic effect upon both the size and yield of product. This is illustrated in Figure 3. At 0.1 *M* KCl, the cDNA was predominantly short (~6.2 S) and similar to that seen in Figure 1. Raising the salt concentration to 0.15 *M* KCl led to a twofold increase in the total quantity of cDNA made, but no increase in the length was observed. Conversely reduction of the KCl concentration to 0.05 *M* resulted in the formation of a larger cDNA product (~8 S). Finally the omission of KCl resulted in the synthesis of even larger cDNA products (~10.5 S) which were long enough to represent transcripts of the complete ovalbumin mRNA. As can be seen in Figure 3, however, there was also a considerable amount of cDNA which was of a smaller size (<6 S). On a number average basis, these represented a fairly large proportion of the synthesized cDNA product. The labeled material near the surface of the gradient was not due to contaminating labeled nucleotides since chromatography of the cDNA product on a Sephadex G-50 column led to an identical sucrose gradient profile. Clearly reducing the salt concentration had somehow facilitated the transcription of mRNA template by a portion of the reverse transcriptase molecules. One possible explanation is that the secondary structure of the mRNA template is an important factor in determining how far along the RNA template the enzyme can transcribe before it is "blocked" by the extensive secondary structure in ovalbumin mRNA (N. T. Van, I. W. Holder, S. L. C. Woo, A. R. Means, and B. W. O'Malley, manuscript in preparation). Reducing the KCl concentration would clearly help in destabilizing large hairpin loops. However, attempts to increase the yield of long cDNAs by decreasing the Mg^{2+} ion concentration were unsuccessful. This resulted in a large loss in both the yield and size of cDNA when concentrations of less than 4 m*M* $MgCl_2$ were utilized. In addition to using a low salt buffer we employed an additional alternative method to destabilize the ovalbumin mRNA molecule by increasing incubation temperature. Accordingly we have observed that by increasing the temperature to 46°C during synthesis of the cDNA and limiting the time of incubation to 5 min, a cDNAs population containing a very high proportion of complete cDNAs could be obtained (Figure 1). Any further increase in the incubation time above 5 min served only to increase the proportion of short cDNAs giving a profile similar to that shown in Figure 3 for the cDNA synthesis with no KCl at 37°C. It is important to note that in this procedure the enzyme was added to a cold (4°C) reaction mixture containing the mRNA and oligo(dT) primer and quickly vortexed, and then the whole reaction mixture was immediately incubated at 46°C. If the enzyme was added initially to the reaction mixture at room temperature or at 46°C, a lower yield of completed cDNAs was obtained. Moreover, temperatures as high as 54°C did not significantly affect the profile shown in Figure 1 for long $cDNA_{ov}$.

Characterization of the Long $cDNA_{ov}$ Copy. We next attempted to characterize this long $cDNA_{ov}$ in order to establish whether the cDNA represented a true complementary copy of the ovalbumin mRNA. The average nucleotide length obtained as shown in Figure 1 was 1681 nucleotides. This long length was confirmed by polyacrylamide gel electrophoresis in 98% formamide using the procedure described by Boedtker et al. (1973). We have shown previously that there is a discrepancy between the length of ovalbumin mRNA determined by electrophoresis and the length determined by a number of other methods such as sedimentation velocity, poly(A) analysis, and electron microscopy (Woo et al., 1975). Ovalbumin mRNA migrates slower than expected (as a 21S species) during electrophoresis in formamide containing polyacrylamide gels, corresponding to a chain length of 2620 nucleotides. In Figure 4a it can be seen that there was a good linear relationship between the electrophoretic mobility of ϕX174 DNA (open form), phage Qβ-RNA, and *E. coli* sheared DNAs 274 NT and 132 NT using the electrophoretic system described above. In Figure 4B is shown the electrophoretic profile of the long $cDNA_{ov}$ preparation described in Figure 1. A weight aver-

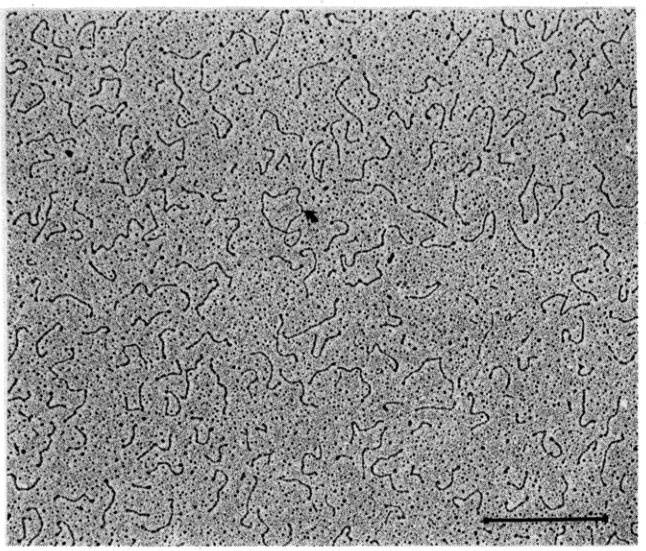

FIGURE 5: Electron microscope analysis of a long $cDNA_{ov}$ preparation. A preparation of $cDNA_{ov}$ was prepared as described in Figure 1. An aliquot of the cDNA was placed on a grid using the formamide modification of the Kleinschmidt technique as described by Robberson et al. (1972). The arrow points to a molecule of circular ϕX174 DNA added to the preparation to obtain an exact estimate of the lengths of the $cDNA_{ov}$. The bar length = 1 μ.

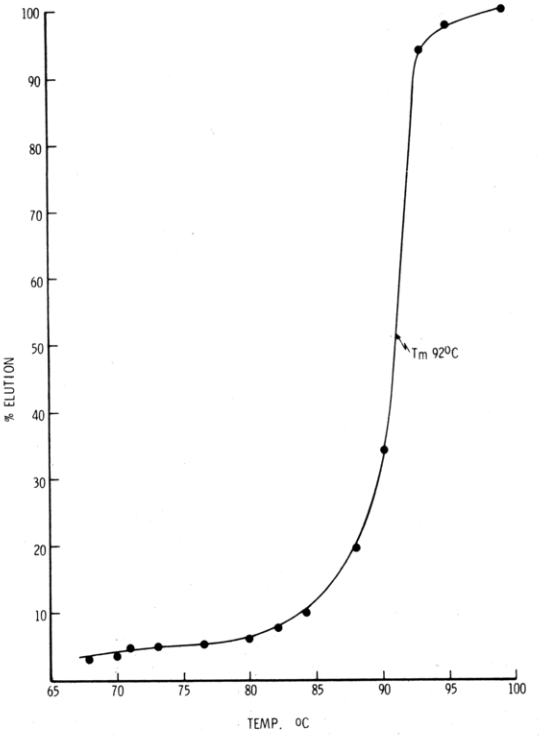

FIGURE 6: Thermal denaturation of long $cDNA_{ov}/mRNA_{ov}$ on a 1-ml hydroxylapatite column. The hybrid was bound to hydroxylapatite in 0.14 *M* phosphate buffer at 50°C. The temperature was raised stepwise. The amount of cDNA eluting in 3 × 2 ml of 0.14 *M* phosphate buffer was monitored at each temperature. Data are plotted on the cumulative percent of cDNA eluted at each temperature.

age polynucleotide length of 1800 NT can be obtained from Figure 4A. This is very close to our estimated length of ovalbumin mRNA which was 1890 ± 180 NT (Woo et al., 1975).

We have also examined the contour length of the long $cDNA_{ov}$ by electron microscopy. A preparation of long $cDNA_{ov}$ similar to that described in Figure 1 was prepared by the formamide modification of the Kleinschmidt technique as described by Robberson et al. (1972). Figure 5 shows that a considerable proportion of the $cDNA_{ov}$ was long. We estimate on a weight average basis a length of 1630 ± 600 nucleotides. The difference between the values obtained on alkaline gradients and that of the formamide gels and electron microscopy is unclear.

Although the $cDNA_{ov}$ was large enough to contain a complete ovalbumin mRNA, its long length might have been partly the result of anomalous synthesis of a homopolymer as described by Falvey et al. (1974) or "slippage" during transcription so that a part of the mRNA was transcribed more than once.

Figure 6 shows the melting profile of a $mRNA_{ov}$/long $cDNA_{ov}$ hybrid. The sharp transition at 92°C was in good agreement with a T_m of 89°C seen when total unique sequence chick DNA was analyzed in the same way (Rosen et al., 1973). The lack of a significant amount of DNA melting below 85°C indicates that very little base pair mismatching had occurred and that the reverse transcriptase had faithfully copied the mRNA. The purified long $cDNA_{ov}$ itself failed to bind to the hydroxylapatite column under the conditions used in this experiment (and those described below). It is therefore unlikely that the melting profile seen was due to melting of one or more intrastrand hairpin loops in the cDNA itself.

In Figure 7 it can be seen that when the long $cDNA_{ov}$ was hybridized to an excess of purified ovalbumin mRNA, the hybridization reaction occurs with a $R_0t_{1/2}$ of 4.62×10^{-3} mol sec $l.^{-1}$ which was lower than the observed $R_0t_{1/2}$ for the short cDNA (6.39×10^{-3} mol sec $l.^{-1}$). This observed reduction in the $R_0t_{1/2}$ was consistent with the excluded volume theory of Wetmur and Davidson (1968), in which the hybridization rate is proportional to the square root of the length of the shorter strand (Wetmur, 1971; Lee and Wetmur, 1972; Hutton and Wetmur, 1973a,b). The cDNA hybridization reaction occurred in both cases within a 100-fold range of mRNA R_0t values and attained a final value of 100% hybridization. This result strongly indicates that the cDNA was transcribed from a population of RNA molecules of similar complexity. We should point out that no exact formula has been derived to date, for DNA–RNA hybridization RNA excess using S_1 nuclease to assay the extent of hybridization. Equation 1 described in the methods section may be an approximation for interpretation of the data. However, while the absolute $R_0t_{1/2}$ values may be slightly in error, the relative difference between the long and short cDNAs will still be valid.

The best evidence that much of the long $cDNA_{ov}$ represented a complete transcript of the ovalbumin mRNA is the ability of the cDNA/mRNA hybrid to totally protect the mRNA against S_1 nuclease. In the experiment shown in Figure 8, increasing amounts of $cDNA_{ov}$ were hybridized with ^{125}I-labeled mRNA. The hybridization reactions were allowed to go to completion in each case. The DNA/RNA hybrid was then treated with S_1 nuclease which will hydrolyze any unhybridized RNA. In theory a 1:1 ratio of cDNA and mRNA should be 100% resistant to S_1 nuclease. As shown in Figure 8, a ratio of about 3:1 was required for the long cDNA to completely protect the [^{125}I]mRNA suggesting that not all the cDNAs represented complete transcripts of the mRNA. It is possible that we had slightly underestimated the amount of mRNA since accurate estimates of the specific activity of the [^{125}I]mRNA were difficult to determine. By contrast, the short $cDNA_{ov}$ was only able to

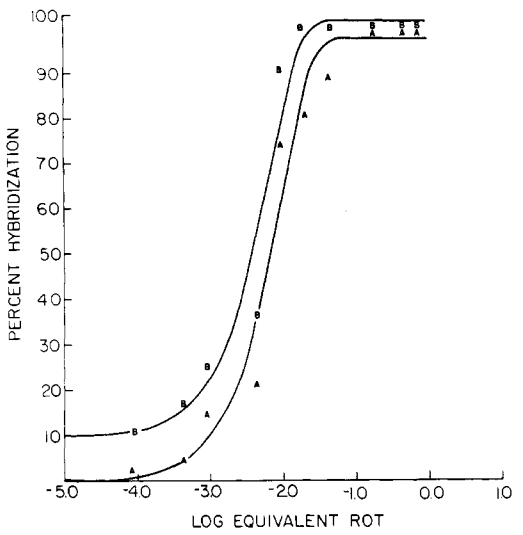

FIGURE 7: Hybridization of $cDNA_{ov}$ with purified ovalbumin mRNA. Varying amounts of ovalbumin mRNA were incubated with 10 ng of $cDNA_{ov}$ at 68°C in 50 μl of 0.6 *M* NaCl, 0.01 *M* Tris-HCl, 1 m*M* Hepes (pH 7.0), and 2 m*M* EDTA to the indicated R_0t values. Hybrid was assayed with S_1 nuclease. Hybridization curves were determined and drawn by computer. (A) Short $cDNA_{ov}$; (B) long $cDNA_{ov}$. R_0t values indicated have been corrected for the effect of salt on the rate of hybridization.

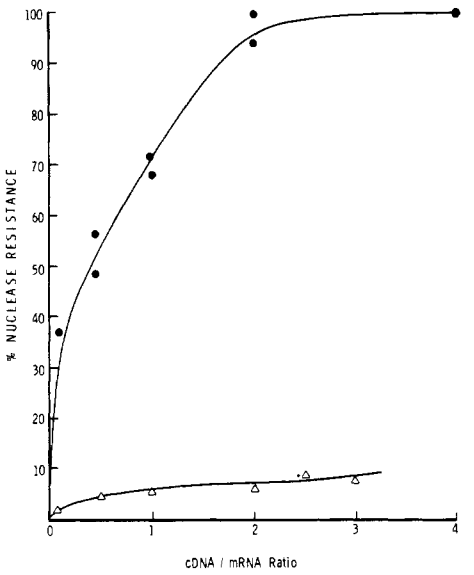

FIGURE 8: Titration of $cDNA_{ov}$ with $[^{125}I]mRNA_{ov}$. Increasing amounts of long (●—●) and short (△—△) cDNA were hybridized to completion with 6 ng of $[^{125}I]$mRNA in 50 μl of 0.6 *M* NaCl, 0.01 *M* Tris-HCl, 1 m*M* Hepes (pH 7.0), and 2 m*M* EDTA. Hybrid was assayed with S_1 nuclease.

protect ~10% of the ovalbumin mRNA over the concentration range examined.

The cDNA preparation described above had a specific activity between 10^5 and 10^6 cpm/μg. This cDNA would be satisfactory for incorporation into eucaryotic cells or plasmids once it was made double stranded by addition of a poly(A) sequence to its 5′-terminal end using terminal deoxynucleotidyl transferase. Then with oligo(dT) and *E. coli* DNA polymerase I the second strand could be copied. For hybridization studies, however, it is desirable to obtain cDNAs of higher specific activity (~10^8 cpm/μg). Efstratiadis et al. (1975) have recently pointed out that low concentrations of dNTPs led to short cDNAs. To obtain long cDNAs of specific activities of about 10^8 cpm/μg, it is clearly desirable to use the highest possible [^{3}H]dNTP concentration in order to avoid the addition of unlabeled dNTPs.

Figure 9A and B shows the effect of different dNTP concentrations upon the size of the synthesized cDNAs. All syntheses of cDNA were carried out in the absence of KCl as described in the legend to Figure 1, except that the concentrations of the dNTPs were varied. In these experiments, [^{3}H]dGTP (20 μCi/ml) was used to label the cDNA product. As can be seen in Figure 9A, the cDNA was very short (~5.5 S) at a substrate concentration of 5 μM for all of the dNTPs including dTTP. Increasing the concentration to 10 and 20 μM increases the size of the cDNA considerably and leads to the synthesis of a minor fraction of long cDNAs. With addition of 30, 50, or 100 μM dNTPs, a large fraction of long cDNA was synthesized. As has been observed by Efstratiadis et al. (1975) and as shown in Figure 9A and B, increasing the concentration of the dNTPs also increases the total amount of cDNA made (when dilution of the [^{3}H]dGTP by increasing amounts of cold dGTP is taken into account). However, in all cases, a considerable fraction of the cDNAs are still short in length. We have observed that the minimum concentration of dNTPs required for synthesis of long $cDNA_{ov}$ was 35 μM (the conditions de-

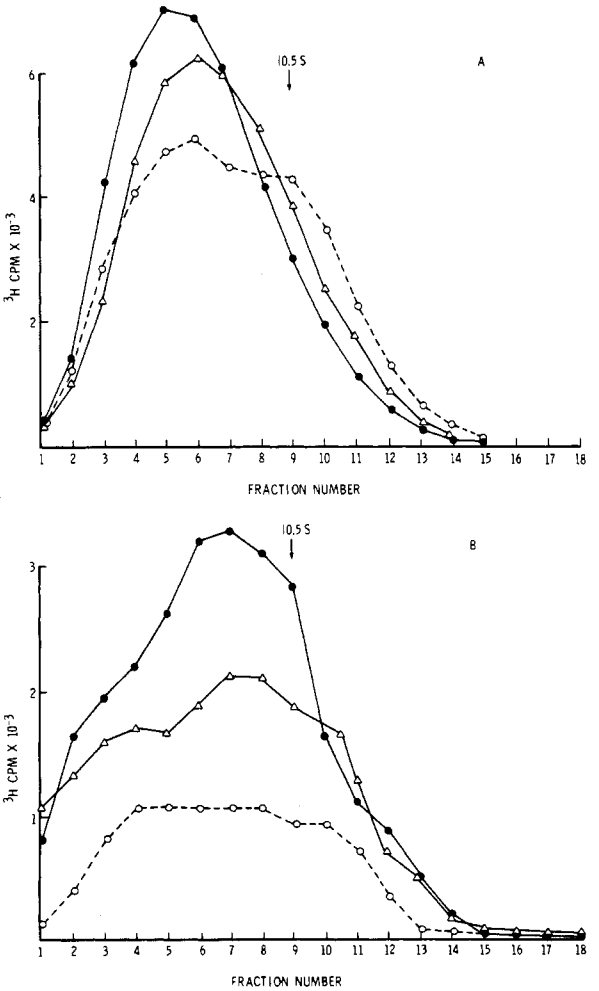

FIGURE 9: Effect of dNTP concentration upon the length of $cDNA_{ov}$. Buffer conditions were the same as those for Figure 1, except that there was no KCl present and the label was [^{3}H]dGTP, 20 μCi/ml. dNTP concentrations were: (A) (●—●) 5 μM, (△—△) 10 μM, and (O- - -O) 20 μM. (B) (●—●) 30 μM, (△—△) 50 μM, and (O- - -O) 100 μM. Synthesis was for 5 min at 46°C.

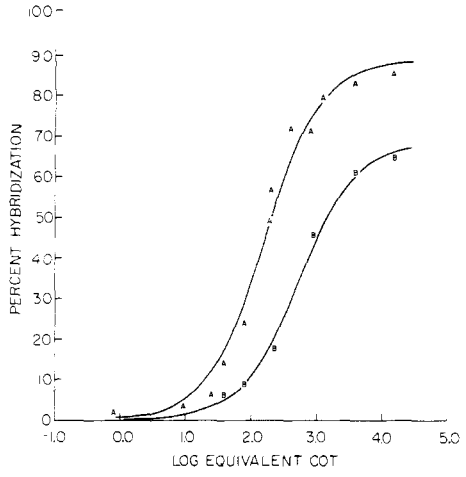

FIGURE 10: Hybridization of (A) long [^{3}H]cDNA and (B) unique sequence chick [^{3}H]DNA to a vast excess of chick DNA. Hybridization was in 50 μl of 0.4 or 0.12 M phosphate buffer and 0.2 mM EDTA for a sufficient time to obtain the required C_0t value. The samples were diluted to 1 ml and analyzed for hybrid using hydroxylapatite. Hybridization curves were determined and drawn by computer. C_0t values indicated have corrected for the effect of salt on the rate of hybridization.

scribed in Figure 1). This population of cDNA thus obtained contained few short cDNAs. Any further reduction in the concentration of dNTPs leads to a dramatic reduction in the size of the $cDNA_{ov}$ product. This is consistent with the report that AMV reverse transcriptase has a K_m for each of the dNTPs between 10 and 30 μM (Leis and Hurwitz, 1974). Nevertheless, the necessity for the addition of a 200 μM concentration of dTTP is unclear.

Hybridization of Complete $cDNA_{ov}$ to Chick DNA. We have shown previously that ovalbumin mRNA reannealed to chick DNA under conditions of vast DNA excess with a $C_0t_{1/2}$ consistent with the interpretation that the ovalbumin gene is represented only once per haploid genome (Rosen et al., 1974). Because of the difficulties in obtaining mRNA of sufficiently high specific activity, and the kinetics of DNA excess hybridization to RNA, it was not possible in those experiments to reach an extent of RNA hybridization greater than 30%. The availability of a long ovalbumin cDNA labeled to a high specific activity of $\sim 10^8$ cpm/μg now allows us to examine the complexity of the entire $mRNA_{ov}$ molecule and to search for small amounts of repetitive sequences which may be present in the ovalbumin mRNA. The cDNA was prepared and purified as described under Materials and Methods. The cDNA was hybridized to a vast excess of chick DNA as described under Materials and Methods. The ratio of $cDNA_{ov}$ to chick DNA was 1:4 $\times 10^6$. Hybridization of the $cDNA_{ov}$ to DNA was assayed at different C_0t values using hydroxylapatite (Rosen et al., 1974). The minimum requirement for a double-stranded DNA structure to bind to hydroxylapatite in 0.12 M phosphate at 60°C appears to be about 50 nucleotides (Wilson and Thomas, 1973). Therefore, if even a small segment (>50 nucleotides) of the mRNA was transcribed from a repeated DNA sequence, the whole cDNA should remain bound to the hydroxylapatite column in 0.12 M phosphate at low C_0t values. Since this interpretation is dependent upon the absence of cDNA degradation during hybridization, we carried out analysis of the cDNA on alkaline sucrose gradients following hybridization to a C_0t of 10000 mol sec l.$^{-1}$. These results showed that there was no degradation of the cDNA during the long term incubation necessary for a complete reaction. The technique is therefore clearly a sensitive test for the presence of repeated DNA sequences in ovalbumin mRNA. As can be seen in Figure 10, the long $cDNA_{ov}$ hybridizes with a $C_0t_{1/2}$ of 159 mol sec l.$^{-1}$. Also shown in Figure 10 is a hybridization curve of unique sequence (~400 nucleotides long) ^{3}H-labeled chick DNA isolated as described previously (Rosen et al., 1973). The $C_0t_{1/2}$ of the unique sequence DNA was 527 mol sec l.$^{-1}$. Clearly, the complete copy $cDNA_{ov}$ hybridized to an excess of chick DNA with a rate approximately three times faster than that of the short unique sequence DNA. As will be discussed below, a slightly faster rate of hybridization of the long cDNA compared to the shorter unique sequence DNA is to be expected because of the effects of length upon the rate of hybridization. The data suggest therefore that there is little if any repetitive sequences present in ovalbumin mRNA.

Discussion

The results of this paper indicate that ovalbumin mRNA can be faithfully transcribed into a complementary single-stranded DNA much of which is approximately the same size as ovalbumin mRNA by AMV reverse transcriptase. The relative amounts of complete and partial mRNA transcripts made were shown to be dependent upon the salt, the concentration of dNTPs, the time, and the temperature of synthesis. Because the addition of purified reverse transcriptase did not cause a reduction in size of a previously synthesized cDNA and since there was no detectable RNase activity associated with the purified enzyme, it seems plausible that short cDNAs made under some conditions are due to failure of the enzyme to transcribe the complete mRNA template. The enzyme may dissociate from the template during cDNA synthesis due to regions of secondary structure present within the $mRNA_{ov}$ molecule. Alternatively, conditions of low salt and high temperature may bring about a conformational change in the enzyme structure such that this new conformation is capable of transcribing an RNA template more effectively. At present we tend to favor the first possibility, namely, that the conformational changes are in the RNA template itself.

Our hypothesis is that the enzyme starts at the 3′-terminal end of the mRNA (using the oligo(dT) primer) and transcribes along the single strand of RNA toward the 5′-terminal end. At one or more points it will encounter hairpin loops (or other form of secondary structure) present in the mRNA (N. T. Van, J. W. Holder, S. L. C. Woo, A. R. Means, and B. W. O'Malley, manuscript in preparation) where there is a high probability that further cDNA synthesis stops. Although this hypothesis remains to be definitely established, it is supported by the observations that in the absence of salt and with an increase in temperature, conditions which help to destabilize mRNA secondary structures, we obtain long cDNA transcripts. It is of interest to note that Efstratiadis et al. (1975) have found that the incomplete cDNA transcripts of rabbit globin mRNA occur as discrete size classes rather than a continuous array of decreasing sized transcripts. This would be consistent with partial blockage or dissociation of the enzyme at discrete sites on the mRNA. These workers also noted similar discrete size classes of partial cDNA transcripts for silkmoth chorion mRNAs, ovalbumin mRNA, and Fibroin mRNA. Ross et al. (1972) have also observed two size classes for rabbit globin short cDNAs. Our failure to obtain

long $cDNA_{ov}$ by reducing the Mg^{2+} concentration (a condition which also favors destabilization of RNA secondary structure) could be explained on the basis that the Mg^{2+} salts of the NTPs are the true substrates for the enzyme and that a concentration of at least 6 m*M* Mg is required for enzyme activity.

As we have previously mentioned, incubations longer than 5 min do not lead to synthesis of more long cDNAs. While the total Cl_3CCOOH precipitable incorporation will continue to increase (even after several hours) this increased synthesis is primarily of short cDNAs. Grandgenett et al. (1973) have presented evidence that the purified AMV reverse transcriptase subunit contains an endogenous RNase H activity. Thus, it may be possible that small RNA fragments liberated from the DNA/RNA hybrid formed during transcription serve as templates for further enzyme synthesis. In any event 5 min would appear to be more than sufficient time for the enzyme to transcribe the mRNA template once, if the rate of transcription is comparable with that of DNA dependent RNA polymerase (Davis and Hyman, 1970). Based on the yield of long cDNA obtained, it appears that only about 20% of the ovalbumin mRNA molecules were actually transcribed into cDNA. While this is high by normal standards, the reason for this limited transcription is unclear. A twofold increase in this enzyme concentration resulted in only about 25% of the mRNA molecules present being transcribed.

The reason why the short $cDNA_{ov}$ can protect only 8-10% of the iodinated mRNA against nuclease (Figure 8) is unclear to us. Possibly the short hybrids are not quite stable to S_1 nuclease. Also a large fraction of the short $cDNA_{ov}$ is composed of poly(dT) sequences which will hybridize with the poly(A) sequences of the $[^{125}I]mRNA_{ov}$. Since most of the ^{125}I-labeled material is in the form of 5-iodocytosine (Commerford, 1971), the apparent percentage of $[^{125}I]mRNA_{ov}$ protected against nuclease will be somewhat lower than that expected based upon the length of the short $cDNA_{ov}$. Clearly more detailed studies on the mode of cDNA synthesis by AMV reverse transcriptase using a natural mRNA template are necessary before the mechanism of transcription is well understood. We should also mention that if the secondary structure of the RNA does play an important role in the mode of action of the enzyme, work with other mRNAs may require different incubation conditions for cDNA synthesis dependent upon that particular mRNAs secondary structure.

The possibility now of transcribing from mRNAs, long cDNAs labeled to high specific activities provides an extremely useful tool for molecular biologists. Of general interest in immunology for example would be the synthesis of long cDNAs complementary to immunoglobulin light chain mRNA to answer the question of antibody diversity (Diggelman et al., 1973; Honjo et al., 1974). As described above, it should also be possible to synthesize long double-stranded cDNA and incorporate such double-stranded DNAs into bacterial plasmids or phage λ (Morrow et al., 1974). Long cDNAs should be of use in mRNA sequence studies (Poon et al., 1974). We have also found that long $cDNA_{ov}$'s yield more precise data about the in vitro transcription of ovalbumin RNA sequences from chromatin and reconstructed chromatin (Harris et al., 1975). This is due to the faster hybridization rate of longer cDNAs (Harrison et al., 1974) and the greater stability of long cDNA-RNA hybrids to S_1 nuclease (Harris et al., 1975). The occurrences of sequences in the long cDNA closer to the operator region of the ovalbumin gene now become available to hybridize to short in vitro chromatin RNA transcripts that arise due to premature transcription termination of the gene. Finally, we have a greater ability to follow the progression of transcription from the site of chain initiation by differential hybridization with long and short $cDNA_{ov}$.

Recent studies suggest that a large fraction of eucaryotic mRNA sequences are transcribed from DNA sequences which occur only once per genome (Davidson and Britten, 1973; Bishop et al., 1972; Harrison et al., 1972; Suzuki et al., 1972; Greenberg and Perry, 1971; Firtel et al., 1972). However, there is also strong evidence that at least some mRNAs are transcribed from repeated DNA sequences. Histone mRNAs are a well-known example (Kedes and Birnstiel, 1971). Campo and Bishop (1974) have suggested that as much as 25% of rat myoblasts mRNAs are transcribed from very moderately (<tenfold) repetitive DNA sequences. Ryffel and McCarthy (1975) recently reported even higher amounts of repetitive DNA sequences transcribed into mouse L-cell mRNAs.

All mRNAs that have been isolated and purified to date are known to contain more nucleotides than are required for coding. These "extra" nucleotides which appear to be present on each side of the coding region (in the few cases that have been examined) have an unknown function (Proudfoot and Brownlee, 1974; Milstein et al., 1974; Perry et al., 1975). Dina et al. (1973) have presented evidence that mRNAs from developing Xenopus embryos are internally heterogeneous, containing a part transcribed from unique DNA and a covalently linked smaller part transcribed from a family of homogeneously repeated sequences. Campo and Bishop (1974) on the other hand found no evidence for such internally heterogeneous mRNAs in rat myoblast tissue culture cells. Long cDNA labeled to high specific activity can yield more detailed information about the sequence complexity of mRNAs. Our hybridization studies of long $cDNA_{ov}$ to an excess of chick DNA is a good example of this. The observed $C_0t_{1/2}$ of the long $cDNA_{ov}$ on hydroxylapatite was 159 mol sec $l.^{-1}$, while that for the unique sequence DNA was 527 mol sec $l.^{-1}$. A faster rate of hybridization of the long $cDNA_{ov}$ should be expected because of its length. It has been shown that for nonrepetitive DNA the rate of reassociation of long DNA fragments with short fragments is faster (by the ratio of their lengths) than is the reassociation of short fragments with each other when assayed with hydroxylapatite (Davidson et al., 1973; Graham et al., 1974). The approximately 3.3-fold faster hybridization rate of the long $cDNA_{ov}$ with the ~400 NT long chick DNA was therefore consistent with the ovalbumin mRNA sequences being represented only once per genome. There was clearly no evidence that the untranscribed region of $mRNA_{ov}$ belonged to a family of repeated sequences.

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Added in Proof

We have recently observed that the absolute concentration of RNA in μg/ml in the reaction mix is also important in determining the final size of the cDNA product. A tenfold reduction in the mRNA concentration lead to a short cDNA product. Reactions involving small amounts of mRNA should therefore be carried out in a small volume using conditions exactly as described above to obtain a long cDNA product.

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