The Ovalbumin Gene

IN VITRO ENZYMATIC SYNTHESIS AND CHARACTERIZATION*

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Using purified single-stranded ovalbumin complementary DNA (cDNAov) as a template for avian myeloblastosis (AM) virus reverse transcriptase, we have enzymatically synthesized a complete double-stranded cDNAov sequence. Our data suggests that many single-stranded cDNAov molecules contain short double-stranded sequences (hairpins) at their 3’ termini capable of acting as primers for synthesis of complete double-stranded cDNAs. Optimum conditions for synthesis of the double-stranded cDNAov were found to be a high temperature (46°C) and a low salt concentration. Nevertheless, in all cases 40% of the initial single-stranded cDNAov molecules fail to prime for synthesis of a complementary double strand. Following synthesis, the second DNA strand is covalently linked to the first cDNAov strand as shown by analysis on alkaline sucrose gradients. The two strands have a high Tm on hydroxyapatite (89°C). These intact double-stranded cDNAov structures have a buoyant density in CsCl gradients of 1.700 g/cm3 and rapidly renature after heat denaturation with a C1/2 value of less than 2 x 10^{-6} mol s liter^{-1}. All size classes of cDNAs, i.e. partial as well as complete transcripts of the mRNA, are capable of forming double-stranded structures. The closed loop of the double-stranded cDNAov could be opened with S1 nuclease. The denatured complementary strands of the cDNAov then renatured with the appropriate second order kinetics at a C1/2 value of 1.89 x 10^{-3} mol s liter^{-1}. Using the enzyme terminal deoxyribonucleotidyltransferase to label to free 3’-terminal end of double-stranded [32P]cDNAov with 3H, we demonstrate a convenient procedure to study the site for restriction endonuclease cleavage within the ovalbumin gene.

Reverse transcription of eukaryotic messenger RNAs using RNA-directed DNA polymerase (reverse transcriptase) has proved exceedingly useful to prepare sensitive and specific hybridization probes for a variety of experiments designed to study gene frequency, mRNA metabolism and transcription of chromatin in uitro (1-10). It is now possible to obtain cDNA preparations that are complete and faithful copies of their respective mRNA templates (11, 12). The availability of such cDNAs makes them attractive candidates for the insertion of eukaryotic structural genes into bacterial plasmids (13-16). The limited sequence heterogeneity of these DNA preparations compared to total (14, 17) or enriched (18) cellular DNA species not only considerably simplifies the problem of detecting bacterial clones containing the required gene sequence but also helps to minimize the unknown potential dangers of cloning large amounts of cellular DNA containing possible undesirable gene sequences. Techniques are available for the insertion of single-stranded cDNAs into bacterial plasmids (19), however better yields of cDNA containing plasmids can be obtained using double-stranded cDNAs as the starting material. Recently Efstratiadis et al. (20) have shown that rabbit globin cDNA synthesized by AM virus reverse transcriptase contains a small double-stranded sequence (hairpin) at the 3’ terminus of the cDNA. The presence of this hairpin appears to allow the globin cDNA to serve as template primer for Escherichia coli DNA polymerase I which can then synthesize the second DNA strand. We demonstrate in this paper that following removal of the mRNA template and actinomycin D, the reverse transcriptase enzyme itself is capable of using the “first strand” of the cDNAov as a template primer to make the complete “second strand” of the structural gene for ovalbumin.

The availability of sequence-specific restriction endonucleases allows sequence-specific fragmentation of double-stranded DNAs. Electrophoresis of these DNA fragments can yield valuable information concerning the location of various base sequences within the DNA molecule. However, for most double-stranded DNAs, it is not normally possible to distinguish the two ends of the DNA, thereby making it impos-

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‡ The abbreviations used are cDNAov, ovalbumin complementary DNA; AM virus, Avian myeloblastosis virus; dNTP, deoxyribonucleoside triphosphate; C1/2, the product of the total DNA concentration in moles of nucleotides/liter and the time in seconds; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid.

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sible to assign restriction enzyme sites without further indirect data. Taking advantage of the fact that one end of the double-stranded cDNA (that complementary to the 5'—terminal end of the mRNA) exists as a closed loop, we can add specifically a single-stranded H-labeled polynucleotide tail to th 3'—terminal end of the double-stranded cDNA. If the double-stranded cDNA is labeled with 32P, it is then possible using agarose gel electrophoresis to unambiguously locate the approximate position of a restriction enzyme site within the DNA molecule. We have used the restriction enzyme Hae III to demonstrate this procedure. The use of a multiplicity of such enzymes should eventually allow the construction of a partial sequence map of the ovalbumin gene. Such a sequence map should greatly facilitate our sequencing of the ovalbumin gene.

**METHODS**

**Isolation of Ovalbumin mRNA**—Purified ovalbumin mRNA was isolated as we have described previously (21, 22). The purity of the ovalbumin mRNA was determined by analytical procedures such as acid-urea agarose gel electrophoresis, polyacrylamide gel electrophoresis in 30% formamide, sucrose gradient centrifugation in 70% formamide, translation in vitro in a heterologous protein synthesizing system, and hybridization followed by hybridization with radioactively labeled DNA and the resultant oligonucleotide maps. These procedures have been described in detail elsewhere (22). Only ovalbumin mRNA of greater than 99% purity was used in these studies.

**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 (23) and will be described in detail elsewhere.1 In summary, purified virions of AM virus 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 on phosphocellulose. The enzyme fractions were eluted from the phosphocellulose column with a linear phosphate gradient. Active fractions of reverse transcriptase were pooled, diluted, and chromatographed on a second phosphocellulose column. The final pool of the 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.

**Renaturation of Double-stranded cDNA**—Renaturation experiments with the cDNA were carried out in tapered reaction vials (Regis Chemical Co., Ill.). During the incubation, the vials were submerged to prevent condensation. The renaturation reaction was performed in a final volume of 50 μl containing: 0.6 mM NaCl, 10 mM Tris/HCl, pH 7.0 at 25°C, 0.001 M Hepes (pH 7.0 at 25°C), 0.002 M EDTA, and 50 mg/ml of Escherichia coli DNA. Samples were initially placed in a boiling water bath for 5 min and then incubated at 68°C for time intervals ranging from 0.1 to 24 hr, depending on the cDNA preparation being tested. After hybridization the vials were quickly frozen in liquid nitrogen. To assay the extent of hybridization, 0.2 ml of a solution containing 0.1 M sodium acetate, 0.8 mM MgCl2, 100 μg/ml of actinomycin D, 1% sodium dodecyl sulfate, and 250 μg/ml of poly(dT) was added to 500 μl of the cDNA sample. 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. The cDNA was resuspended in 300 μl of 0.2 M sodium acetate and ethanol to remove most of the ribonucleotides. We wish to emphasize the importance of monitoring the extent of ethanol precipitation of the cDNA in the above steps and those described below. 

**Addition of [3H]poly(dT) to the 3'-Terminal of Double-stranded cDNA**—It has been shown recently that calf thymus terminal deoxynucleotidyltransferase efficiently polymerizes pyrimidine deoxyribonucleoside triphosphates in the presence of Co2+ using the free 3'-terminal (OH) on each polydeoxynucleotide as a primer (25, 26). We have found Co2+ more efficient than Mg2+ for adding [3H]poly(dT) to the 3'-terminal end of double-stranded cDNA. Under optimum conditions, reaction rates as high as 60 dT residues added/oh were observed. Several conditions for the synthesis were as follows: 250 μl of [3H]poly(dT) was lyophilized to dryness and resuspended in 100 μl of a solution containing 60 mM Tris/HCl (pH 7.5), 35 mM KCl, 500 μM CoCl2. The final concentration of [3H]poly(dT) was adjusted to 290 μM. After the addition of 10 μg of double-stranded [15N]DNA in 55 μl of H2O (blended on a Vortex mixer) to the reaction mixture followed by 50 units of terminal deoxynucleotidyltransferase (P-L Biochemicals, Inc.) the reaction mix was incubated at 37°C. The course of reaction was monitored by removing 1-μl aliquots, precipitating with trichloroacetic acid, and counting the “H and 15N labeled trichloroacetic acid-insoluble material. The reaction was terminated by the addition of EDTA to 1 mM and sodium acetate to 0.1 M followed by precipitation of the DNA with ethanol. The cDNA was then precipitated from the aqueous phase with ethanol as described above.

**Centrifugation of cDNA in Alkaline Sucrose Gradients**—Samples of cDNA to be analyzed were treated with ethanol, redissolved in 0.1 M of a mixture of 0.5 N NaOH and 0.5 N NaCl, and layered onto an 8 to 18% linear sucrose gradient in the same solution. The gradient was centrifuged for 24 hr at 38,000 rpm at 5°C in a Beckman SW 50 rotor. Fractions were neutralized and counted in Aquasol. Sheared E. coli DNA (3.8 S and 5.1 S) as well as the linear (15.1 S) and the circular form (18.4 S) of α214 DNA were used as markers. The S values of the cDNA 4S, 2S, and 1S of cDNA is the S value of the 0.14T4 DNA. Approximately 5% of the cDNA was precipitated with ethanol. The S values of the open and closed forms of α214 DNA in alkali have been previously reported by Studier (27).
Synthesis of Ovalbumin Gene

S1 Nuclease Nicking of Double-stranded cDNA<sub>α</sub>—The closed loop of the double-stranded cDNA<sub>α</sub> was nicked open by treating 3 μg of the cDNA<sub>α</sub> (in 300 μl of 0.3 M sodium acetate, pH 4.5, 0.6 M NaCl, 4 mM ZnCl<sub>2</sub>, and 50 μl/ml of E. coli DNA) with 100 μl of S1 nuclease (1 mg/ml) for 3 h at 37°. The cDNA was then extracted with chloroform and precipitated with ethanol.

S1 Nuclease Assay of Double-stranded cDNA<sub>α</sub>—The extent to which the reverse transcriptase transcribed the single-stranded cDNA<sub>α</sub> into double-stranded cDNA<sub>α</sub> was assayed essentially as described above for the DNA renaturation studies. However, we have observed that the transcriptase enzyme itself is able to confer some protection of single-stranded cDNA<sub>α</sub> to S1 nuclease digestion. Therefore, all cDNA preparations were routinely extracted with chloroform (after adding E. coli DNA carrier) and precipitated with ethanol before carrying out the S1 assay on the cDNA.

Slab Gel Electrophoresis—The size of the cDNA<sub>α</sub> was determined by electrophoresis on an agarose slab gel. Varying concentrations of agarose were dissolved in 50 mM Tris (pH 8.4), 20 mM sodium acetate, 18 mM sodium chloride, and 2 mM EDTA. Electrophoresis was in the same buffer for 4 to 6 h at 100 mA/slab. The gel apparatus (16 x 16 cm) was made by Biight Craftline (Cold Spring Harbor, New York). The gel was stained with 5 μg/ml of ethidium bromide in the electrophoresis buffer for 10 to 20 min. The DNA was visualized with ultraviolet light.

All cDNA<sub>α</sub> species were eluted from the agarose gels using the “freeze-squeeze method” of Thuring et al. (28).

Restriction Enzyme Digestion of cDNA—The restriction endonuclease, Hae III, was obtained from BRL Research Laboratories, Inc. (Bethesda, Md.) and was assayed in 6 mM Tris/HCl (pH 7.4), 6 mM MgCl<sub>2</sub>, and 6 mM 2-mercaptoethanol. Reactions were performed at 37° for 2 h with 1 unit of enzyme/5 μg of cDNA<sub>α</sub>. To each cDNA preparation, 1 μg of phage λ DNA was added to monitor the enzyme activity.

RESULTS

Synthesis of Double-stranded cDNA<sub>α</sub>—A number of investigators have shown that cDNA made by reverse transcriptase contains double-stranded regions (29-34). In some (29) but not all (33, 34) cases it has been demonstrated that the double-stranded regions rapidly regain resistance to single-stranded nuclease after denaturation. A 3'-terminal hairpin structure has been proposed to exist in rabbit globin cDNA by Efstratiadis et al. (20). They have utilized this structure for the synthesis of a double-stranded molecule using Escherichia coli DNA polymerase I.

In an analogous manner, we have used highly purified AM virus reverse transcriptase to synthesize the second strand of cDNA<sub>α</sub>. This enzyme has the advantage that it does not possess the 5'- or 3'-terminal endonuclease activities present in E. coli DNA polymerase I, thereby eliminating the possible dangers of exonuclease hydrolysis of the template strand during the 4-h synthesis procedure.

We have recently shown that AM virus reverse transcriptase is capable of working efficiently in the presence of high temperature (46°) and low monovalent cation concentration to synthesize single-stranded cDNA from a mRNA template using an oligo(dT) primer (12). Synthesis under these conditions appears to favor the formation of cDNAs that are complete copies of the mRNA template. It now appears that these conditions are also optimal for the synthesis of double-stranded cDNA<sub>α</sub> structures. When the enzyme was incubated with the same amount of cDNA<sub>α</sub> (without actinomycin D) at 37° in the presence of 0.1 M NaCl or at 46°, an almost 2-fold greater yield of double-stranded product was obtained at the higher temperature (Fig. 1). In this experiment the amount of cDNA converted to the double-stranded form was estimated on the basis of the specific activity of the [3H]GTP and single-stranded [3H]cDNA used. Evidence that the products were indeed double-stranded is given below. The data in Fig. 1 is in marked contrast to the synthesis of the initial complete single-stranded cDNA<sub>α</sub> from its mRNA template since that reaction takes place within a few minutes (12). Clearly, the reaction for the synthesis of the second strand takes place very slowly. The reason for this is unclear to us. Similar slow rates for the synthesis of double-stranded globin cDNA using E. coli polymerase I have been observed (20).

Perhaps the hairpin loop is unstable and only transiently folds back on the DNA to act as a primer.

We should also stress that the time required for the synthesis of the original single-stranded cDNA<sub>α</sub> from mRNA<sub>α</sub> was only 5 min at 46°. We have shown previously that longer incubation times lead to the synthesis of greater proportions of incomplete cDNA transcripts (12). Our preliminary observations indicate that such cDNA preparations are less efficient templates for double-stranded cDNA<sub>α</sub> synthesis. We have also observed that neither poly(dT)<sub>12-18</sub> nor poly(dA)<sub>12-18</sub> primers stimulated the rate or extent of synthesis of the second strand. This observation as well as additional evidence presented below rules against the possibility of an internal poly(A) sequence binding to the poly(dT) segment of the 5'-terminal of the single-stranded cDNA<sub>α</sub>., thereby serving as a primer for the reverse transcriptase.

Characterization of Double-stranded cDNA<sub>α</sub>—We next attempted to characterize the cDNA<sub>α</sub> duplex in order to establish that it is a complete double-stranded structure. Fig. 2 illustrates the sedimentation profile of single-stranded and double-stranded cDNA<sub>α</sub>, on a 8 to 18% alkaline sucrose gradient. The single-stranded cDNA<sub>α</sub> sediments with a peak sedimentation value of 10.5 S corresponding to a DNA molecule ~1750 nucleotides long. The double-strand cDNA<sub>α</sub>, on the other hand, sediments in a broad band with a peak at 14 S corresponding to a DNA molecule ~3500 nucleotides long. In this experiment only the initial strand of the double-stranded cDNA<sub>α</sub> contained 32P. Clearly, the only way the 10.5 S single-stranded cDNA<sub>α</sub> could be converted to the 14 S material is by the covalent attachment of further nucleotides to the 10.5 S cDNA. This could occur either via the attachment of a random nucleotide sequence possibly a homo-

![Fig. 1. Effect of temperature and NaCl concentration upon the extent of double-stranded cDNA<sub>α</sub> synthesis using single-stranded cDNA<sub>α</sub>, as a template and primer, with AM virus reverse transcriptase.](http://www.jbc.org/)
polymer or via the attachment of a second complementary copy of the first strand to the 3'-terminal of that molecule. The presence of the latter was confirmed in the following experiments.

A preparation of single-stranded [32P]cDNA was used as a template for the synthesis of double-stranded cDNA with [3H] in the second strand using the conditions described under "Methods." This cDNA was then incubated with S1 nuclease under conditions in which all single-stranded DNA would be hydrolyzed. In Fig. 3, it can be seen that after 2 h of digestion essentially all the single-stranded cDNA was hydrolyzed. In marked contrast only 44% of the [32P] in the double-stranded cDNA preparation was made trichloroacetic acid-soluble. This indicates that the majority of the [32P] in the first strand of the cDNA was converted to a duplex form. It is interesting to note that almost 100% of the [3H] incorporated into the second strand of the cDNA was resistant to S1 (Fig. 3), again suggesting the formation of a double-stranded structure.

We were disappointed that not all of the original [32P]cDNA was converted to a double-stranded form. There were clearly two extreme possibilities. Either all of the single-stranded [32P]cDNA molecules were made partially double-stranded or only a fraction of the molecules were completely converted to a double-stranded form. To distinguish between these two possibilities we analyzed the double-stranded [32P]/[3H]-labeled cDNA by hydroxylapatite chromatography. The minimum requirement for a double-stranded DNA structure to bind to hydroxylapatite in 0.12 M phosphate at 60° appears to be 50 nucleotides (35). We have shown previously that single-stranded (complete) cDNA fails to bind to hydroxylapatite under these conditions (12). Therefore, any cDNA that have greater than approximately 50 nucleotides added as a double-stranded structure during the second incubation with reverse transcriptase will be retained on hydroxylapatite and will only be eluted with 0.4 M phosphate. We observed that 40% of the [32P]cDNA was eluted with 0.12 M phosphate while 60% was eluted with 0.4 M phosphate. The 0.4 M phosphate material also contained 98% of the [3H] counts. When the [32P]cDNA present in the 0.12 M phosphate fraction was reacted with S1 nuclease, 95% of it was digested by S1 nuclease within 2 h (Fig. 3). In marked contrast to this was the [32P]cDNA eluted in 0.4 M phosphate. In this instance, essentially all the DNA was resistant to S1 nuclease.

Further evidence that the double-stranded cDNA eluted from hydroxylapatite with 0.4 M phosphate did in fact contain two perfectly matched strands in a duplex form was illustrated by its Tm of 89° on hydroxylapatite (Fig. 4) and by its buoyant density in CsCl after isopycnic gradient centrifugation. Fig. 5 shows the distribution of [32P]-labeled double-stranded and [3H]-labeled single-stranded cDNA on such a gradient. The G + C content of the double-stranded cDNA should be 41% (22). Using the relationship (36) $p_{DS} = 1.66 + 0.098 (X_G + X_C)$, where $p_{DS} =$ buoyant density of a double-stranded DNA, $X_G$ and $X_C =$ mole fraction of G and C, we calculate a buoyant density of 1.700 g/cm3. This theoretical calculation is in excellent agreement with our experimentally observed density determination.

Because the double-stranded cDNA contains a loop at one end we would expect it to rapidly renature after heat denaturation with zero order kinetics. This is in fact what was observed. When the cDNA duplex purified on hydroxylapatite was...
heat-denatured and allowed to renature to a C\textsubscript{T} value of only 2 \times 10^{-4} \text{ mol s liter}^{-1}, 98\% of the cDNA returned to the double-stranded form as assayed by hydroxylapatite chromatography.

The enzyme S\textsubscript{T} nuclease is able to digest single-stranded and loop structures (20). When the double-stranded cDNA\textsubscript{ov} purified by hydroxylapatite was treated with S\textsubscript{T} nuclease as described under “Methods,” the hairpin loop on the cDNA was opened. Upon denaturation and renaturation the cDNA\textsubscript{ov} strands displayed second order renaturation kinetics as shown in Fig. 6. The observed C\textsubscript{T} value was 1.89 \times 10^{-3} \text{ mol s liter}^{-1}.

The hybridization reaction went to completion indicating that all the denatured cDNA was capable of forming a double-stranded structure hybridization. By a number of different criteria therefore, it appears that the cDNA\textsubscript{ov} retained on hydroxylapatite in 0.12 \text{ M} phosphate is a double-stranded cDNA structure with a hairpin loop at one end and free 5'- and 3'-ends at the other end.

To determine whether all lengths of single-stranded cDNA\textsubscript{ov} can prime for double-stranded cDNA synthesis, we carried out the experiment shown in Fig. 7. A preparation of \textsuperscript{32}P-labeled single-stranded cDNA\textsubscript{ov} was fractionated on an alkaline sucrose gradient as described in Fig. 2. The single-stranded cDNA\textsubscript{ov} in each fraction was each resuspended in the buffer described under “Methods” for double-stranded cDNA synthesis. The extent of double-stranded cDNA\textsubscript{ov} synthesis was assayed by the incorporation of \textsuperscript{3}H\textsuperscript{d}GTP into trichloroacetic acid-insoluble material and is shown by the histograms above. Synthesis was for 4 h at 46\° as described in Fig. 1.

![Figure 5](http://www.jbc.org/)  
**Fig. 5.** CsCl equilibrium density gradient centrifugation of double- and single-stranded cDNA\textsubscript{ov}. — —, \textsuperscript{3}H-labeled single-stranded cDNA\textsubscript{ov}; ■■■■, \textsuperscript{3}P-labeled double-stranded cDNA\textsubscript{ov}; ●●●●, optical density profile of Escherichia coli DNA; ▲—▲, density of CsCl solution. A 5-ml solution of CsCl in 0.01 M Tris/HCl (pH 8.0), 10 mM EDTA was adjusted to a density of 1.725 g/cm\textsuperscript{3} and centrifuged in a Beckman 65 rotor at 30,000 rpm for 56 h at 15°.

![Figure 6](http://www.jbc.org/)  
**Fig. 6.** Renaturation of denatured double-stranded cDNA\textsubscript{ov}. Varying amounts of heat-denatured (S\textsubscript{T} nuclease-treated) \textsuperscript{3}P-labeled double-stranded cDNA\textsubscript{ov} were incubated at 68\° in 50 \mu l of 0.6 M NaCl, 0.01 M Tris/HCl, 1 mM Hepes (pH 7.0), and 2 mM EDTA to the indicated C\textsubscript{T} values. Hybrid was assayed with S\textsubscript{T} nuclease. Hybridization curves were determined and drawn by computer. The C\textsubscript{T} values indicated have been corrected for the effect of salt on the rate of hybridization.

![Figure 7](http://www.jbc.org/)  
**Fig. 7.** An assay to determine whether all lengths of single-stranded cDNA\textsubscript{ov} are capable of acting as template and primers for the synthesis of double-stranded cDNA\textsubscript{ov}. A preparation of \textsuperscript{3}P-labeled single-stranded cDNA\textsubscript{ov} was fractionated on an alkaline sucrose gradient as described in Fig. 2. The single-stranded cDNA\textsubscript{ov} in each fraction (●●●●) was precipitated with ethanol. These cDNA\textsubscript{ov} precipitates were each resuspended in the buffer described under “Methods” for double-stranded cDNA synthesis. The extent of double-stranded cDNA\textsubscript{ov} synthesis was assayed by the incorporation of \textsuperscript{3}H\textsuperscript{d}GTP into the particular cDNA\textsubscript{ov} structures were completely double-stranded. We have also attempted to determine if the rate limiting step in the formation of double-stranded cDNA\textsubscript{ov} was the initiation of a double-stranded structure or the completion of the second strand. A preparation of single-stranded \textsuperscript{3}P-labeled cDNA\textsubscript{ov} was prepared as described under “Methods.” The total DNA preparation was deproteinized with chloroform precipitated with ethanol and fractionated by gel electrophoresis in 2% agarose.
stranded cDNAs, respectively, after electrophoresis and autoradiography in 2% agarose gels. The size distribution of double-stranded cDNA, molecules after synthesis at 46°C for 10, 30, 90, and 300 min under conditions similar to those described in Fig. 1 is shown in Fig. 8, B, C, D, and E, respectively. The only major double-stranded cDNA, structure present at any time was the complete double-stranded cDNA,. Partial double-stranded structures were not seen even after only 10 min of synthesis. These observations therefore suggest that the rate-limiting step in the formation of a double-stranded cDNA, structure was the initiation of its synthesis rather than the completion of the double-stranded structure.

Isolation of Double-stranded cDNA, for Sequence Studies—The 32P-labeled double-stranded cDNA, structures have several potential uses for DNA sequence studies. However, before carrying out these studies it would be convenient to label one end of the DNA with 3H. We have chosen to use the enzyme terminal deoxyribonucleotidyltransferase to add [3H]-dTTPs to the free 3'-OH of each 32P-labeled double-stranded cDNA, molecule. The best yield of such a structure was obtained using the 32P-labeled double-stranded cDNA, prepared as described above. The preparation was not exposed to hydroxylapatite chromatography and thus contains 40% single-stranded DNA and 60% double-stranded DNA. The specific activity of the DNA was 106 cpm/μg. Approximately 10 [3H]-dT residues were added to each 3'-terminal end of the DNA in the preparation. A number average length of 1000 nucleotides was assumed using the procedure described under "Methods."

The total DNA preparation was then deproteinized with chloroform, precipitated with ethanol, and fractionated by gel electrophoresis in 2% agarose (Fig. 9). The location of [32P]-cDNA, could be monitored by autoradiography and the size determined relative to the plasmid ColEl and SV40 marker DNA fragments created by digestion with the restriction enzyme Hpa I. A distinct band corresponding to 1750 ± 60 nucleotide base pairs could be observed. Other double-stranded bands at 1650 ± 60, 1200 ± 50, and 1150 ± 50 nucleotide base pairs were also seen (Fig. 9). The regions of the gel corresponding to 1750, 1650, 1200, and 1150 nucleotide base pairs were excised from the gel and collected.

Treatment of Double-stranded cDNA, with Restriction Endonuclease Hae III—The 32P-labeled double-stranded cDNA, species 1750, 1650, 1200, and 1150 nucleotide base pairs were run on a second 4% agarose gel. It can be seen in Fig. 10, B, D, F, and H, that each cDNA species migrated as a single component with an electrophoretic mobility in agreement with its size observed in Fig. 9. Pretreatment of the 1750 nucleotide base pairs-long cDNA, with the restriction endonuclease Hae III as described under "Methods." resulted in its cleavage into two species, 1120 ± 50 and 790 ± 40 nucleotide base pairs long, as shown in Fig. 10A. Over 98% of the 3H label was in the 1120 nucleotide base pairs fragment (i.e., this fragment contained those sequences which code for the 3'-terminal end of the mRNA). This suggests that the Hae III restriction nuclease site was located approximately 790 nucleotides from those sequences coding for the 5'-terminal end of the mRNA in the ovalbumin gene (Fig. 11).

Treatment of the 1650-nucleotide base pairs double-stranded cDNA, species with the Hae III resulted in three cleaved DNA species, 1120 ± 50, 790 ± 40, and 650 ± 40 nucleotide base pairs long (Fig. 10C). Our simplest expla-

![Fig. 8. Electrophoresis of single- and double-stranded 32P-labeled cDNA, on 2% agarose gels. The single-stranded 32P-labeled cDNA, (A) was the same cDNA, as that described in Fig. 2. A distinct band of complete transcripts approximately ~1750 nucleotides long was seen by autoradiography. This region of the gel was excised and used as template for double strand synthesis. The length of the high specific activity 32P-labeled double-stranded cDNA, synthesized after 10, 30, 90, and 300 min is shown in B, C, D, and E, respectively. Purified 32P-labeled 1750 nucleotide base pairs (NTP)-long double-stranded cDNA, migrated as shown in F. Purified 32P-labeled 1750 nucleotides (NT) long single-stranded cDNA, migrated as shown in G. Electrophoresis was for 4 h at 100 m.A/slab.](http://www.jbc.org/)
could then use the double-stranded loop region as a primer for
synthesis of the cDNA duplex. Activities of this nature have
been shown to occur in vitro for this enzyme (37). The fact that
Efstratiadis et al. (20) observed little loss of ³²P from the
first strand of cDNA during the second strand synthesis would
require at least in their case that any unhybridized 3'-terminal
segment be small. It could be argued that this may not be a
general case. The fact that no such 3'- to 5'-terminal exonu-
clase has been reported to be present in purified AM virus
reverse transcriptase would suggest that the above mechanism
is not true. We suggest that the enzyme in the course of its
synthesis of the first cDNA strand either stops after the
completion of a structure that is either a hairpin or loop in the
mRNA (i.e. before transcribing the next adjacent single-
stranded region) or, alternatively, it may after transcribing the
cDNA fold back on the cDNA, thereby transcribing a short
segment of the cDNA as a double-stranded structure. These
possibilities probably will only be resolved by accurate se-
quencing of a number of different cDNA species in the future.

Our observation that the optimum condition for double-
stranded cDNA synthesis was not a high NaCl concentration
at 37° but rather a low salt concentration at 46° was somewhat
surprising to us. One might have expected that the former
conditions would help stabilize a short hairpin loop thereby
making it easier for the enzyme to find a primer for double
strand synthesis. Possibly some of the as yet unknown factors
that allow the enzyme to transcribe the mRNA in its entirety
at high temperatures are important for synthesis of the second
strand of the cDNA.

The procedure we have used to label the free 3'-terminal end
of the double-stranded cDNA will allow us to conveniently
map the position of restriction endonuclease specific sequences
within the gene. We have illustrated this with the restriction
enzyme Hae III. The availability of now more than two dozen
of these enzymes, many of which have six-nucleotide-long
specific sequence requirements, will not only allow us to
determine the approximate location of these sequences within
the gene, but will yield homogenous short double-stranded
DNA fragments suitable for sequencing studies.

The in vitro enzymatic synthesis of the ovalbumin structural
gene should greatly facilitate our studies on the organization
and expression of the gene in vivo. We have recently suc-
sceeded in integrating the gene prepared as described above into
a bacterial plasmid. The DNA sequences obtained from suitable
clones of plasmid containing cells should yield amounts of
ovalbumin gene DNA far in excess of what can be obtained by
reverse transcriptase alone as described.

With the observations that double-stranded cDNAs can be
made from rabbit globin and now from chick ovalbumin
mRNAs, it is likely that the procedures described in these two
papers can be applied to many if not all mRNA preparations.
The availability of cDNAs from complex mixtures of mRNAs
should allow, in the future, the molecular cloning of not only
any specific gene sequences, but also the cloning of tissue-
specific (39) and hormone-specific gene sequences (2).

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