The Ovalbumin Gene

IN VITRO ENZYMATIC SYNTHESIS AND CHARACTERIZATION*

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Using purified single-stranded ovalbumin complementary DNA (cDNA) as a template for avian myeloblastosis (AM) virus reverse transcriptase, we have enzymatically synthesized a complete double-stranded cDNA sequence. Our data suggests that many single-stranded cDNA 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 cDNA were found to be a high temperature (46°C) and a low salt concentration. Nevertheless, in all cases 40% of the initial single-stranded cDNA molecules fail to prime for synthesis of a complementary double strand. Following synthesis, the second DNA strand is covalently linked to the first cDNA strand as shown by analysis on alkaline sucrose gradients. The two strands have a high Tm on hydroxylapatite (89°C). These intact double-stranded cDNA structures have a buoyant density in CsCl gradients of 1.700 g/cm3 and rapidly renature after heat denaturation with a Cd value of less than 2 x 10^-3 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 cDNA could be opened with S1 nuclease. The denatured complementary strands of the cDNA then renatured with the appropriate second order kinetics at a Cd 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]cDNA 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 vitro (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.1 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 cDNA 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-

1 Unpublished observations.
2 The abbreviations used are cDNA, ovalbumin complementary DNA; AM virus, Avian myeloblastosis virus; dNTP, deoxyribonucleo-
side triphosphate; Cd, the product of the total DNA concentration in moles of nucleotides/liter and the time in seconds; Heps, 4-(2-hydroxy-
yethyl)-1-piperazine ethanesulfonic acid.
against 50% glycerol, 0.2 M potassium phosphate (pH 7.2), 2 mM
pool of the reverse transcriptase fraction was concentrated by dialysis
rechromatographed on a second phosphocellulose column. The final
containing 0.4 M sodium acetate (pH 4.5), 0.8 M NaCl, 5 mM ZnCl,
the phosphocellulose column with a linear phosphate gradient. Active
tested. After hybridization the vials were quickly frozen in liquid
clarified crude extract was passed through a DEAE-column. Reverse
and will be described in detail elsewhere.3 In summary, purified virions
should greatly facilitate our sequencing 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 electrophore-
sis in 7% formamide, sucrose gradient centrifugation in 70% formam-
hide, translation in vitro in a heterologous protein synthesizing system, and polynucleotide
followed by probe-directed homochromatography of 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 myelo-
blastosis virus reverse transcriptase was generously supplied by Dr. J.
W. Beard (Lifo 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 KCI. The clarified crude extract was passed through a DEAE-column. Reverse
transcriptase was batch-eluted from the DEAE-column and adsorbed
onto a phosphocellulose column. The enzyme fractions were eluted from the phosphocellulose column with a linear phosphate gradient. Active fractions of reverse transcriptase were pooled, dialyzed, and stored
at -20°C. Renaturation of Double-stranded cDNA,,-Renaturation exper-
iments with the cdNA were carried out in tethered reaction vials
(Rexis 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 M NaCl, 10 mM
Tris/HCl, 0.001 M Heps (pH 7.0 to 25°C), 0.002 M EDTA, and 60 μg/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 0.4 M sodium acetate (pH 4.5), 0.8 M NaCl, 5 mM ZnCl,
and 150 μg of the pooled S1 nuclease peak from the final DEAE-cellulose
column (6) was added to each vial. The vials were incubated for 2 h at
37°C. The S1 nuclease resistant DNA was precipitated with 2% trichloroacetic acid, collected on Millipore filters and dried. The filters
were then dissolved in 3 ml of "Cellusolve" (ethylene glycol mono-
methyl ether) by shaking for 2 h at room temperature and counted in
10 ml of Aquasol. Both the CdNA and the DNA renaturation curves were determined using a computer designed to fit a curve
containing the data and defined by the equation d/Do = P [1
- ((Cd/Cd0) + 1) - 1]/B where d/Do = the fraction of renatured DNA;
P = final extent of hybridization; B = background or zero time
hybridization; C = mol s liter-1 of nucleotides DNA. Details of the computer
procedure are described in detail elsewhere (24).

Preparation and Purification of the cDNA—A number of oligonucleotides corresponding to specific activity of 50,000 cpm/μg
were synthesized by deoxynucleotidyl transferase using the following
conditions. One millicurie of 3H-yttrium trifluoromethane sulfonic acid
(5 μCi/ml) was added to 5 ml of H2O (blended on a Vortex mixer) to the reaction mixture followed by 50
units of terminal deoxynucleotidyltransferase (P-L Biochemicals, Wisc.). The reaction was incubated at 37°C. The course of reaction was monitored by removing 1-μl aliquots, precipitating with
trichloroacetic acid, and counting the 3H- and 32P-labeled trichloro-
acetic acid-insoluble material. The reaction was terminated by the addition of 10 μl of double-stranded [32P]EDTA in 50 μl of H2O

 Addition of [H]Poly (d(T)) to the 3′-Terminal of Double-stranded cDNA,,-It has been shown recently that calf thymus terminal
deoxynucleotidyltransferase efficiently polymerizes pyrimidine deox-
yribonucleoside 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 "H poly(dT) to
3′-terminal ends of double-stranded cDNA. Under optimal conditions,
reactions rates as high as 60 dT residues added/hr can be obtained. The
conditions for the synthesis were as follows: 250 μl of [H] Poly(dT)
was lyophilized to dryness and resuspended in 100 μl of a solution
containing 60 μM Tris/HCl (pH 7.5), 35 mM KCl, 500 μM CoCl2.
The final concentration of the [H] Poly(dT) was adjusted to 250 μM. After
the addition of 10 μg of double-stranded [32P]EDTA in 50 μl of H2O

Centrifugation of cDNA in Alkaline Sucrose Gradients—Samples of cDNA to be analyzed were precipitated with ethanol, redissolved
in 0.1 M of a mixture of 0.1 M NaOH, 10 μg/ml NaCl, and 5 mM EDTA,
and layered onto an 8 to 18% linear sucrose gradient. The gradients were centrifuged for 24 h at 38,000 rpm at 5°C in a Beckman SW 40 rotor. Fractions were neutralized and counted in
Aquos. Sheared E. coli DNA (3.8 S and 5.1 S) as well as the linear (16.1 S) and the circular form (18.4 S) of 34S DNA were used as markers. The S values (S;::,!~) of the E. coli DNA (27) were measured
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 34S DNA in alkalai have been previously reported by Studier (27).
Synthesis of Ovaalbumin Gene

S. 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 mM 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 S<sub>1</sub> nuclease (1 mg/ml) for 2 h at 37°. The cDNA was then extracted with chloroform and precipitated with ethanol.

S<sub>1</sub> 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 S<sub>1</sub> 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 S<sub>1</sub> 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 5 h at 100 mA/slab. The gel apparatus (16 x 16 cm) was made by Blair Craftline (Cold Spring Harbor, New York). The gel was stained with 5 μl/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 soaked in 50 mM Tris/HCl (pH 7.4), 6 mM NaCl, 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>. 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 contain 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 specific 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' and 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 ([3P]c)DNA 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 48°. 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 effective templates for double-stranded cDNA<sub>α</sub> synthesis. We have also observed that neither poly(dT)12-18 nor poly(dA)12 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 3' -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 represented a complete double-stranded structure. Fig. 2 illustrates the sedimentation profile of single-stranded and double-stranded cDNA<sub>α</sub> on a 5 to 18% alkaline sucrose gradient. The single-strand cDNA<sub>α</sub> sediments with a peak sedimentation value of 10.5 S corresponding to a DNA molecule ~1750 nucleotides long (27). The double-strand cDNA<sub>α</sub> on the other hand sediments as a broad band with a peak at 14 S corresponding to a DNA molecule ~3500 nucleotides long (27). 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-strand 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-
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 $^{32}$P-cDNA$_{av}$ was used as a template for the synthesis of double-stranded cDNA$_{av}$ with $^3$H in the second strand using the conditions described under "Methods." This cDNA was then incubated with S$_1$ 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$_{av}$ was hydrolyzed. In marked contrast only 44% of the $^3$P in the double-stranded cDNA$_{av}$ preparation was made trichloroacetic acid-soluble. This indicates that the majority of the $^3$P in the first strand of the cDNA was converted to a duplex form. It is interesting to note that almost 100% of the $^3$H incorporated into the second strand of the cDNA$_{av}$ was resistant to S$_1$ (Fig. 3), again suggesting the formation of a double-stranded structure.

We were disappointed that not all of the original $^{32}$P-cDNA$_{av}$ was converted to a double-stranded form. There were clearly two extreme possibilities. Either all of the single-stranded $^{32}$P-cDNA$_{av}$ 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 $^{32}$P/$^3$H-labeled cDNA$_{av}$ 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$_{av}$ fails to bind to hydroxylapatite under these conditions (12). Therefore, any cDNA$_{av}$ molecules 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 $^{32}$P-cDNA$_{av}$ 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 $^3$H counts. When the $^{32}$P-cDNA$_{av}$ present in the 0.12 M phosphate fraction was reacted with S$_1$ nuclease, 95% of it was digested by S$_1$ nuclease within 2 h (Fig. 3). In marked contrast to this was the $^{32}$P-cDNA$_{av}$ eluted in 0.4 M phosphate. In this instance, essentially all the DNA was resistant to S$_1$ nuclease. On the basis of these results it would appear therefore that the synthesis of the second strand of cDNA$_{av}$ from a single-stranded cDNA$_{av}$ template is an all or none effect. Either the synthesis goes to completion for each molecule or it fails even to initiate. Longer incubation times for the synthesis of the double-stranded cDNA$_{av}$ failed to improve the yields of duplex material.

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

Because the double-stranded cDNA$_{av}$ 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$_{av}$ duplex purified on hydroxylapatite was...
heat-denatured and allowed to reanneal 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{1} 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{1} 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 renaturation 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 (\textcircled{1}) was precipitated with ethanol. These cDNA\textsubscript{ov} precipitates were resuspended in a 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]dGTP into trichloroacetic acid-insoluble material and is shown by the histograms above. Synthesis was for 4 h at 46\degree C as described in Fig. 1.

For the reaction to go to completion. We demonstrated in Fig. 3 that after this length of time any double-stranded 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{32}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 (Fig. 8A). The predominant molecular weight cDNA\textsubscript{ov} species corresponding to a complete DNA transcript of the mRNA (\sim 1750 nucleotides) was excised from the gel and used as template for double-stranded cDNA\textsubscript{ov} synthesis. Double-stranded cDNA\textsubscript{ov} structures migrate slower than their corresponding single-stranded DNA counterparts in this electrophoresis system. Fig. 8, G and F, shows the position of purified \textsuperscript{32}P-labeled complete transcript single-stranded and double-
stranded cDNA, 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, molecule. 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 deoxynucleotidyltransferase 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 10^6 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 seen. Other double-stranded bands at 1650 ± 60, 1200 ± 50, and 1150 ± 50 nucleotide base pairs were also seen (Fig. 9). The region 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 rerun 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 of the mRNA). This suggests that the Hae III restriction enzyme site was located approximately 790 nucleotides from those sequences coding for the 5'-terminal end of the mRNA in the ovalbumin gene (Fig. 11).

The 1120-nucleotide base pairs-long cDNA, species is summarized in Fig. 11. With the use of a number of other restriction enzymes this method should allow us in the future to do extensive mapping of restriction enzyme sites within the ovalbumin gene. This work is presently in progress.
Our observation that reverse transcriptase is capable of using single-stranded cDNA as a primer and a template supports the observation of Efstratiadis et al. (20) who demonstrated that E. coli DNA polymerase I can make a double-stranded cDNA structure using rabbit globin single-stranded cDNA as a template. The E. coli DNA polymerase I, however, possesses a 3'→5' exonuclease activity (37). It was possible in those studies that the single-stranded cDNA possessed internal hairpin loops. Loops of this nature are known to exist in mRNAs (38). The exonuclease activity could have hydrolyzed unhybridized sequences near the 3' terminal end of the cDNA back to internal loop sequences. The polymerase 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'→5' terminal exonuclease 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 sequencing 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°C but rather a low salt concentration at 46°C 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 succeeded 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 tissuespecific (39) and hormone-specific gene sequences (2).

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