Synthesis of Full Length cDNAs from Four Partially Purified Oviduct mRNAs*

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We have employed sequential AMVirus* reverse transcriptase, Escherichia coli DNA polymerase I and S1 nuclease reactions to synthesize double-stranded cDNAs1 (9, 10). Each reaction has been optimized with the focus of obtaining predominantly full length, double-stranded copies in good yield.

EXPERIMENTAL PROCEDURES

Materials—Most of the reagents and animals have been described previously (11, 12). Materials were obtained as follows: sodium heparin from Riker Laboratories; formamide (99%) from Matheson, Coleman and Bell; barbituric acid from Mallinckrodt; AG 50 1-8 mixed bed resin from Bio-Rad Labs; 3H-deoxycytidine triphosphate (22.8 Ci/mmol) from the International Chemical and Nuclear Corp. and 3H-deoxyguanosine triphosphate from Schwarz/Mann (7.3 Ci/mmol); deoxyguanosine 3'H-triphosphate from New England Biolabs; 3'Hideoxyctydine triphosphatase (22.8 Ci/mmol); deoxyguanosines 3'H-triphosphatase from New England Nuclear (114 to 137 Ci/mmol) and Amersham/Searle (150 to 250 Ci/mmol). Purified reverse transcriptase (Lot G-1176, 28,216 units/mg, and Lot G-577, 73,829 units/mg) were supplied by Dr. J. W. Beard (Life Sciences, Inc., St. Petersburg, Fla.).

Generously supplied as gifts were mouse globin mRNAs from Dr. Henry Barr, Department of Biological Sciences, Stanford University; E. coli mRNA from Dr. Frank Lee, Department of Biological Sciences, Stanford University; SV40 DNA from Steve Goff and Mike Goldberg, Department of Biochemistry, Stanford University Medical School; E. coli DNA polymerase I from Dr. Arthur Kornberg, Department of Biochemistry, Stanford University Medical School, and Drosophila 3'HJRNA from Dr. Gray Craigie, Department of Biological Sciences, Stanford University.

Preparation of Polysomal RNA—Polysomes were prepared as described previously (13) or by a modification of that method developed for preparing larger amounts of RNA. An 8-g piece of laying hen oviduct magnum, fresh or stored in liquid nitrogen, was passed through a diethylpyrocarbonate-treated Harvard tissue press just prior to homogenization. The ground oviduct was scraped into a dounce homogenizer containing 40 ml of 5% sucrose in TNMH. Homogenization and centrifugation were carried out as in Ref. 3. Twenty milliliters of the decanted postmitochondrial supernatant were layered onto a discontinuous sucrose gradient containing 13.4 ml of m sucrose in TNMH over 4 ml of 7.5 % sucrose in TNMH. Three

1 The abbreviations used are: AMVirus, avian myeloblastosis virus; TNMH, 25 mm Tris(Cl), pH 7.6, 25 mm NaCl, 5 mm MgCl2, 1 mg/ml of sodium heparin; SBT, 10 mm Tris(Cl), pH 7.4, 5 mm EDTA, 1% sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid; SDS, sodium dodecyl sulfate; dXTPs, deoxynucleotide triphosphates.

2 D. Kemp and D. Hogness, unpublished results.

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homogenizations provided 120 ml of 20% homogenate, enough for six tubes in an SW 27 rotor (Beckman). Centrifugation was at 27,000 rpm for 9 h at 4°C. Long homogenization treatments up to 5 h did not alter polyosome yield. After centrifugation, the opalescent polysome band at the boundary of the two sucrose layers was removed in about 5 ml of sucrose by puncturing the side of the tube with a sterile syringe 5 mm below the band. Polysomes were extracted with phenol/chloroform (1:1) immediately in atom in liquid nitrogen. For deproteinization polysomes were diluted into at least 3 volumes of SBT buffer before extraction. The aqueous phase was re-extracted twice and the RNA made 0.2 M NaCl and precipitated with 2.5 volumes of ethanol at -20°C overnight.

RNA was pelleted at 12,000 × g for 10 min at 4°C. Contaminating DNA, tRNA, and heparin were removed by washing RNA pellets with 3 × sodium acetate, pH 7.4, as described in Method IV in Ref. 13. From 50 to 55% of polynuclear RNA/g of ovuduct were obtained with either the SW 41 (11) or SW 27 rotor. No preferential loss of small polysomes occurred using the SW 27 relative to the SW 41 as judged by polysome profiles on linear 5 to 20% sucrose gradients and by the ratio of translational activities for ovalbumin and ovomucoid mRNAs in polysomal RNA.

Preparation of Poly(A)-containing RNA - Precipitated RNA was washed with 70% ethanol, dried by lyophilization, and dissolved in 15% SDS. Concentrated stock solutions were added to make the RNA 15 A₄₅₀/ml in binding buffer lacking NaCl (10 mM Heps, pH 7.4, 1 mM EDTA, 1% SDS) (11). The RNA was heated at 68°C for 10 min, then chilled in an ice bath. Sufficient 4 M NaCl was added to make the final concentration 250 mM. RNA was chromatographed on polyacrylamide gel (5 ml volume) as described previously (11) with two exceptions: after washing beds with a low salt buffer (11) columns were washed with binding buffer containing 20% formamide (10 bed volumes). Bound RNA was eluted with 98% formamide (10 bed volumes). Bound RNA was eluted with 98% formamide rather than 70%. From 1 to 1.5% of polysomal RNA was recovered in the bound fraction, which was enriched for translational activity and RNA sequence (52). The amounts of enzyme and oligo(dT)₆₀ were noted) at 10 units/µg of RNA. The amounts of enzyme and oligo(dT)₆₀ were saturating. After incubating 1 h at 42°C reactions were terminated by chilling on ice and adding 50 µl of 10 mM Tris/Cl, pH 8, and 10 µl of unlabeled 100 mM DCTP. Forty micrograms of E. coli tRNA was added as carrier. Reactions were extracted with 400 µl of chloroform and chromatographed on Sephadex G-150 in water. Columns with 6 ml bed volumes were prepared in 5-ml plastic pipettes, plugged with siliconized glass wool. The void volume was made 200 mM NaCl and 2.5 volumes of ethanol were added. Recovery of ovalbumin cDNA after chromatography was 90 to 99%.

ST Nuclease Assay - Assays were carried out exactly as described previously (15). ST nuclease was prepared and stored at -20°C as described previously (15).

Protein Electrophoresis - Sodium dodecyl sulfate-acrylamide tube gels were prepared and electrophoresed essentially as described by Laemmli (20). After electrophoresis, gels were sliced into 2-mm slices, each slice incubated with 1 ml of 30% hydrogen peroxide for 3 min (22) prior to addition of 7 ml of 6 M urea solution fluid, Triton X-100/sucrose (13) containing 0.5% Pernamul.

Nucleic Acid Electrophoresis - Electrophoresis of RNA or DNA in agarose gels containing methylmercury hydrosulfite was as described by Bailey and Davidson (21). Gels were prepared by dissolving an appropriate weight of agarose (2% for polysomal RNA gels) in distilled water, heated at 68°C for 3 min (22) prior to addition of solid sucrose and bromphenol blue. Tube gels were cast, in plastic tubes (6 cm long glass plates). Electrophoresis was usually overnight at 15 V followed by several hours at 50 V (constant voltage), until the bromphenol blue was within 1 cm of the bottom. For staining with ethidium bromide, gels were placed in 0.5 M NH₄OH containing 20% formamide gels were heated at 68°C for 10 min immediately before loading to minimize aggregation (15). Polyacrylamide column columns were washed with binding buffer containing 20% formamide (10 bed volumes). Bound RNA was eluted with 98% formamide (10 bed volumes). Bound RNA was eluted with 98% formamide rather than 70%. From 1 to 1.5% of polysomal RNA was recovered in the bound fraction, which was enriched for translational activity and RNA sequence (52).

Preparation of Complementary DNA - All reactions were carried out exactly as described previously (15). ST nuclease was prepared and stored at -20°C as described previously (15).

Electrophoretic Elution - Electrophoretic elution of RNA from acrylamide gel slices was carried out essentially as described by Grunstein and H chick (25).

Autoradiography - CH₂OH agarose slab gels were frozen and coated with Ilford M-1 t6 film, at 20°C. Then the film was developed in a Kodak X-Omat automatic processor.
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have been determined on 3.7% acrylamide gels in the presence of 99% formamide to be 208,800 ± 43,870 and 235,000 ± 28,000, respectively (29).

DNA molecular weight markers were prepared by restriction of circular SV40 DNA with HindIII restriction nuclease in 6 mm Tris/Cl, pH 7.5, 50 mm NaCl, 6 mm MgCl₂, 1 μg/ml of bovine serum albumin, and 80 μg of DNA/ml for 1 h at 37°C. This reaction was then added to a vial containing lyophilized deoxynucleotidetriphosphates both labeled and unlabeled, so that the final concentration of each was 5 μM. E. coli DNA polymerase I was added at 2 units/μg of DNA and the reactions incubated for 1 h at 15°C (30). SV40 [32P]DNA fragments were prepared for electrophoresis in the same manner as cDNA. Molecular weights of the HindIII fragments of SV40 DNA were initially determined by Dana et al. (31) in combined HindIII and -III digests. The specific HindIII fragment lengths can be found in Ref. 32.

Acid Precipitations and Scintillation Counting—Small samples (less than 100 μl) were added to 1 ml of water containing 50 μg of calf thymus DNA (Sigma). An equal volume of cold 10% trichloroacetic acid containing 1% sodium pyrophosphate was added and the mixture kept on ice for at least 15 min. Precipitates were collected onto Millipore (0.45 μm) or GFC filters using a Millipore filtration apparatus. Filters were washed with 30 to 30 ml of cold 5% trichloroacetic acid containing 0.5% sodium pyrophosphate, placed in scintillation vials, and 10 ml of scintillation mixture added. In experiments using 14C, filters were soaked in 1 ml of 0.1 M NaOH for about 30 min at 80°C before being added to scintillation mixture.

RESULTS

The fully estrogen-stimulated hen oviduct is highly specialized. Most of its protein synthesis is devoted to the four eggwhite proteins, ovalbumin, conalbumin, ovomucoid, and lysozyme which comprise approximately 64%, 12%, 8.5%, and 1.5% of soluble protein synthesis, respectively (3). Since these four proteins differ substantially in size from one another (see Table II), we anticipated that centrifugation of oviduct total poly(A)-containing RNA through sucrose gradients would resolve the four mRNAs. Poly(A)-containing RNA was sedimented through an isokinetic 5 to 20% sucrose gradient and the A260 pattern monitored. The resulting profile, Fig. 1a, shows four discrete peaks with the relative abundances and migrations expected for the four mRNAs.

The mRNA activities were identified by translating each gradient fraction in vitro and immunoprecipitating the various products separately. The gradient shown in Fig. 1 was collected into 18 fractions. Each was ethanol-precipitated, resuspended in water, and an equal aliquot used to program a rabbit reticulocyte lysate translation system as described under "Experimental Procedures." [3H]-labeled reaction products were precipitated with either anti-ovalbumin, anti-conalbumin, anti-ovomucoid, or anti-lysozyme (insufficient anti-lysozyme was available for this experiment). As shown in Fig. 1b, the mRNA translation activities for conalbumin, ovalbumin, and ovomucoid are coincident with the appropriate absorbance peak.

Two preparations of enriched mRNA fractions were made. Total poly(A)-containing RNA was sedimented as described under "Experimental Procedures" and the peak fractions for each activity pooled. After concentration by ethanol precipitation, pooled fractions were centrifuged on identical gradients either three times (Experiment 1) or once (Experiment 2). While the additional purification in Experiment 1 simplified subsequent mRNA characterization, a single recenter-}

The synthesis of double-stranded cDNA. Repeated centrifugation was particularly useful for separating ovalbumin and conalbumin mRNAs which sometimes migrated as faster sedimenting aggregates (see "Experimental Procedures").

Partially purified mRNAs from the two experiments were used as templates in the rabbit reticulocyte system. Each directed protein synthesis in a manner consistent with a partial purification for the expected species and substantial separation from the other three (Table I). The sample was heated in SET for 10 min at 68°C before centrifugation. Centrifugation was for 7 h at 20,000 rpm in a Beckman SW 41 rotor. The gradient was collected into 18 fractions which were ethanol-precipitated, washed with 70% ethanol, dried, and resuspended in 100 μl of water. In Panel b each fraction was translated in vitro in the unmodified reticulocyte lysate. The vertical axes represent the radioactivity immunoprecipitable per total lysate. In the case of ovalbumin (OV) , conalbumin (CON), ovomucoid (MU), and lysozyme mRNA, since that purified fraction is enriched 17-fold for lysozyme translational activity in vitro. The nearest neighboring mRNA fraction, ovomucoid, shows little lysozyme translational activity. Second, each peak displays an increased specific activity for the protein's translation in vitro with a fold enrichment consistent with its rate synthesis in vivo. Finally, purification of each peak results in a large decrease in the activity of other specific translationable mRNAs. Expressed as per cent contamination (see legend to Table I) it appears that no fraction is contaminated by any of the other three more than 10%.

The mRNA fractions from Experiment 2 were assessed for purity by translation in the mRNA-dependent reticulocyte lysate and gel electrophoresis in formamide. The reticulocyte lysate was rendered mRNA-dependent by hydrolysis of endogenous mRNA with calcium-dependent micrococcal nuclease which was subsequently inactivated by the addition of EDTA (18, see "Experimental Procedures"). This treatment reduced
synthesis due to endogenous mRNA about 300-fold to 3700 cpm/10 μl and allowed a 5- to 40-fold stimulation of total incorporation with ovoduct mRNA preparations. Portions of each lysate were used to determine the immunoprecipitable and total acid-precipitable radioactivity. The percentage of synthesized protein which was immunoprecipitable was then calculated. The degree to which this percentage represents the physical mRNA purity depends in each case on several parameters: (i) the relative translational activities of all the messenger species present; (ii) differential losses of in vitro synthesized proteins; and (iii) the ability of antibody to precipitate quantitatively the in vitro product. Intrinsic differences in the in vitro translational activities of oviduct mRNAs have been demonstrated previously (8).

Eighty-six per cent of the incorporation in the ovalbumin mRNA-programmed lysate was immunoprecipitable with anti-ovalbumin. The accompanying gel profile (Fig. 2b) of total protein synthesized shows a single peak, co-migrating with authentic ovalbumin plus smaller molecular weight peptides. These faster migrating species are diminished in amount in the total in vitro product from more highly enriched ovalbumin mRNA (95% immunoprecipitable under the same conditions).5

Approximately one-third of the stimulated incorporation in lysates programmed with ovomucoid or lysozyme mRNA fractions was immunoprecipitable for their respective proteins (Fig. 2, d and e). The products of ovomucoid mRNA-directed reactions display a broad peak migrating at 22,000 and lower molecular weight peptides. Ovomucoid purified from eggwhites migrates at about M, = 40,000 on the same gel system, but is at least 25% carbohydrate (33) M, = 22,000 is in good agreement with the expected peptide molecular weight of ovomucoid based on the probable number of amino acids per molecule.6 Lysozyme mRNA-directed incubations show a major peak which co-migrates with authentic lysozyme and a smaller peak, as yet unidentified at about 12,000.

Lyseate programmed with conalbumin mRNA has a low percent precipitability with anti-conalbumin, 19%. Approximately the same proportion of the total protein synthesized co-migrates with authentic conalbumin (Fig. 2c) while a second peak co-migrates with ovomucoid. This test underestimates the purity of conalbumin mRNA due to its relatively poor translatability in this system.7

Large amounts of ovalbumin, ovomucoid, and lysozyme mRNAs from Experiment 2 were electrophoresed on 3.5% acrylamide, 98% formamide gels (Fig. 3). Ovalbumin mRNA (Fig. 3a) displays a major band with a migration slower than 18 S rRNA and leading zone approximately coincidental with 18 S. The faster migrating region probably contains both rRNA and smaller mRNAs which may code for the lower molecular weight peptides seen in Fig. 2b. Ovomucoid mRNA consists of a single central band superimposed on a general background fluorescence. Lysozyme mRNA appears similar, with respect to background, lower in apparent molecular weight and also includes a second prominent band, L2,7 near the leading boundary. At least two factors contribute to the apparent heterogeneity in ovomucoid and lysozyme mRNAs. First, each is probably contaminated by other mRNAs which co-migrate on sucrose gradients as suggested by translation studies (Table II). Second, poly(A) would be expected to broaden each mRNA peak as demonstrated for globin mRNA (36). Insufficient conalbumin mRNA from Experiment 2 was obtained for this test. The ethidium bromide fluorescence from a small amount of conalbumin mRNA prepared in Experiment I is shown in Fig. 3c. A single band is observed migrating more slowly than ovalbumin mRNA.

The prominent, central bands in gels of ovalbumin, ovomucoid, and lysozyme mRNAs were identified as the species in question by translation. The main band from the gel of each mRNA was excised and electrophoretically eluted (see "Experimental Procedures"). RNAs were partially freed from contaminating gel material by phenol extraction and oligo(dT)-cellulose chromatography and each was translated in vitro in the unmodified reticulocyte lysate. The incorporation into immunoprecipitable proteins was RNA concentration-dependent (Fig. 3d). Specific activities are not directly comparable to those given in Table I, since translational inhibitors elute from the gel and are not completely eliminated by our procedure. Gel-eluted ovalbumin mRNA was also translated in the modified reticulocyte lysate; 95% of the total incorporation was immunoprecipitable with anti-ovalbumin.

**Determination of RNA Molecular Weights**

Several systems for gel electrophoresis of nucleic acids in denaturing media have been devised. We explored barbital-buffered formamide-acrylamide (29) and methylmercury-agarose (21) for their suitability in molecular determinations of RNA. The formamide system suffers from the inadequacies of formamide as a denaturant (37) as well as from alterations in relative mobility due to ionic inhomogeneities. Previous work (21, 37) has suggested that CH3HgOH can fully denature RNAs, even those with high guanosine plus cytosine content. Denaturation is dependent on the concentration of CH3HgOH in the gel. To determine a suitable amount of denaturant for our purposes, a small amount of poly(A)-containing RNA was mixed with E. coli 18 S rRNA and electrophoresed on 1.2% agarose tube gels containing increasing concentrations of CH3HgOH. The amount of mRNA applied was such that only ovalbumin RNA ethidium bromide fluorescence was visible. With increasing CH3HgOH each species' mobility decreased due to loss of secondary structure (21) until a plateau mobility was reached (Fig. 4). As seen in the inset, ovalbumin mRNA reached a lower plateau retardation (H/1H,) than the RNAs, consistent with its having a lower guanosine plus cytosine content (38) and less secondary structure. The main panel shows the apparent molecular weight of ovalbumin mRNA calculated from the relative mobilities. By these criteria, 20 mM CH3HgOH appears to be fully denaturing.

Each of the four partially purified mRNAs from Experiment 2 were mixed with suitable markers and electrophoresed in the presence of 20 mM CH3HgOH. Calibration lines were drawn and molecular weights determined for the prominent bands in each mRNA (Fig. 5). Globin mRNAs were not used to determine the line since their molecular weights are known only by other electrophoretic methods (29), but indicate that migration was directly related to log molecular weight in that size range.

The calculated mRNA lengths in nucleotides are given in Table II. Each mRNA contains a considerable number of extra

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5 G. Crouse, unpublished results.
6 L. Kato, W. J. Kohr, and M. Laskowski, Jr. (1977) personal communication.
7 L2 has been seen in three separate preparations of lysozyme mRNA and contains a sufficient number of nucleotides (560 bases, see Table II) to encode avidin which has 128 amino acids. The small peak in Fig. 2c may represent the in vitro translation product of L2.
8 M. Wickens, unpublished results.
mRNA fractions prepared in Experiments 1 and 2 translated in vitro in reticulocyte lysate protein-synthesizing system

TABLE I

mRNA prepared in Experiment 1 was translated in 100-µl reactions (0.2 to 1.0 µg per reaction) and mRNA prepared in Experiment 2 was translated in 250-µl reactions (0.2 to 3.0 µg per reaction). Specific activities are given in "H counts per min immunoprecipitable per µg of input mRNA. At least two concentrations of mRNA were tested for each fraction and all immunoprecipitations were done in duplicate. The cross-translational activities in Part b were obtained in an identical manner with immunoprecipitations carried out for the expected contaminants. Percent contamination was derived as follows: (specific activity of contaminant)/(specific activity of contaminant in total poly(A)-containing RNA) x % soluble protein synthesis in vivo = % cross-contamination. The per cent soluble protein synthesis serves as an approximation of the proportion of total mRNA which each of the four mRNAs comprises. The numbers employed here are stated under "Results." This assumption is reasonable since the amounts of conalbumin, ovalbumin, and ovomucoid have been shown to reflect the level of their translatable mRNAs (7, 33). Contaminating tRNA in the total poly(A)-containing RNA preparations may also influence apparent cross-contaminations; no correction for its presence was included. A sample calculation follows. In this case, the amount of ovalbumin mRNA in the conalbumin mRNA enriched fraction from Experiment 1 is estimated. (2,770 cpm/µg)/(65,530 cpm/µg) x 64% of soluble protein synthesis = 2.7%.

a. Translation of fractionated mRNAs

| Specific activities | Total poly(A)-containing RNA | Fractionated mRNA | Enrichment |
|---------------------|-----------------------------|------------------|------------|
| Ovalbumin           |                             |                  |            |
| Experiment 1        | 65,530                      | 115,470          | 1.8        |
| Experiment 2        | 51,720                      | 110,180          | 2.1        |
| Conalbumin          |                             |                  |            |
| Experiment 1        | 770                         | 5,440            | 7.1        |
| Experiment 2        | 650                         | 3,960            | 6.1        |
| Ovomucoid           |                             |                  |            |
| Experiment 1        | 4,190                       | 31,460           | 7.5        |
| Experiment 2        | 3,340                       | 39,840           | 11.9       |
| Lysozyme            |                             |                  |            |
| Experiment 1        | 200                         | 3,420            | 17.1       |
| Experiment 2        | 390                         | 6,060            | 15.5       |

b. Cross-translation assayable contaminants

| Specific activity | Specific activity | Specific activity | Specific activity |
|------------------|------------------|------------------|------------------|
| Ovalbumin        |                  |                  |                  |
| Experiment 1     | 170              | 2.8              | 280              | <1.0            | N.D.* |
| Experiment 2     | 150              | 2.7              | 580              | <1.0            | N.D.  |
| Conalbumin       |                  |                  |                  |
| Experiment 1     | 2,770            | 2.7              | 360              | <1.0            | N.D.  |
| Experiment 2     | 4,020            | 4.9              | 580              | <1.0            | N.D.  |
| Ovomucoid        |                  |                  |                  |
| Experiment 1     | 1,970            | 1.9              | 340              | <1.0            | N.D.  |
| Experiment 2     | 3,200            | 3.0              | N.D.             | 310              | 1.3   |
| Lysozyme         |                  |                  |                  |
| Experiment 1     | 920              | <1.0             | <0.1             | 420              | <1.0  |
| Experiment 2     | 720              | <1.0             | N.D.             | 2510             | 6.4   |

* N.D. = not done.

nucleotides not required to code for the polypeptide. Assuming an average of 50 3'-adenylate residues (11), there remain approximately 900 additional noncoding nucleotides in ovalbumin and conalbumin mRNAs and 150 in ovomucoid and lysozyme mRNAs. Polypeptide precursors of ovomucoid and lysozyme have recently been identified (35) and are taken into account in Table II. Ovalbumin and conalbumin may be synthesized as slightly larger precursors which could account for a small number of the apparently noncoding nucleotides.

Synthesis of cDNA from Total Poly(A)-containing RNA

Large amounts of total poly(A)-containing RNA were electrophoresed on 98% formamide, 3.5% acrylamide gels and stained with ethidium bromide. The resulting fluorescence pattern displays a prominent band of ovalbumin mRNA and a fainter band of conalbumin mRNA (Fig. 6a). These mRNAs were identified by their migration relative to purified fractions and to 23 S and 16 S rRNAs (Track 2, Fig. 6a). No prominent band of ovomucoid or lysozyme mRNA can be distinguished even though these mRNAs co-migrate with major peaks of optical absorbance in total poly(A)-containing RNA centrifuged on sucrose gradients (Fig. 1). The inability to visualize discrete bands of ovomucoid or lysozyme mRNA in the electrophoretic profile of total oviduct mRNA can be attributed to at least two possible factors: proportionally greater broadening of bands by poly(A) on small mRNAs and the presence of small RNA fragments which remain intact during centrifugation in SET. The electrophoretic profile of cDNA synthesized from total poly(A)-containing RNA (Fig. 6b) is consistent with these interpretations and suggests that not all RNAs in Fig. 6a are templates for reverse transcriptase. Prominent bands are seen at 1,900, 830, and 630 bases, the sizes expected for full length ovalbumin, ovomucoid, and lysozyme cDNAs, respectively.
Fig. 2. Translation of the four mRNA fractions in the messenger-dependent reticulocyte in vitro protein-synthesizing system. 0.9 µg of each mRNA fraction prepared in Experiment 2 were translated in 90-µl reactions. Duplicate aliquots were removed after reaction termination for determination of immunological and acid (5 %)-precipitable incorporation. Percentages given in the figure refer to the ratio of these two quantities. The translational specific activities obtained were 153,000 cpm/µg of ovalbumin (OV) mRNA, 4,900 cpm/µg of conalbumin (Con) RNA, 22,000 cpm/µg of ovomucoid RNA, and 6,900 cpm/µg of lysozyme (Lys) RNA. The remaining reaction mixture was made one time in SDS sample buffer by the addition of a 2-fold concentration stock solution, boiled for 2 min and applied to 10% acrylamide-laemmli gels. After electrophoresis gels were washed for 24 h in 7% acetic acid plus 5% methanol (1 liter per gel with two changes) to remove free label, then sliced. Slices were dissolved and counted as described under "Experimental Procedures." Marker proteins, ovalbumin, conalbumin, lysozyme, and cytochrome c (Cytc) from horse heart were electrophoresed on a parallel gel. [3H]Folate reductase from cultured mouse cells (fol. red., approximately M, = 20,000) was included on all gels.

The relative intensities of full length cDNAs suggest that reverse transcriptase does not copy all of the mRNAs with equal efficiencies. Although conalbumin mRNA is evident in Fig. 2, no full length conalbumin cDNA is visible in the autoradiogram (Fig. 6b). Conalbumin mRNA must therefore be a poor template for synthesis of full length cDNA relative to the other three mRNAs. As shown in Figs. 10 and 12, only a small proportion of the cDNA made from the partially purified conalbumin mRNA fraction is full length.

Parameters Affecting Synthesis of cDNA

Effect of Time and Temperature - The object of the following experiments was to optimize conditions for the synthesis of full length cDNA from each mRNA fraction. Previous work with ovalbumin mRNA template suggested that short incubations with AMVirus reverse transcriptase at 46°C give a maximal yield of full length ovalbumin cDNA (39). When we examined the time course of ovalbumin cDNA synthesis at 37°C, 42°C, and 46°C little effect of temperature on the total incorporation after 1 h was observed (Fig. 7). The reaction was rapid and increasing the incubation temperature from 37-46°C accelerated the initial rate. However, the length distribution of fully denatured ovalbumin cDNA made at these temperatures was indistinguishable for 1-h incubations (Fig. 8,
TABLE II

| mRNA Sequence | Apparent mRNA length | Known coding bases | Probable non-coding bases |
|---------------|----------------------|--------------------|---------------------------|
| Ovalbumin     | 2000                 | 1100^a             | 900                       |
| Conalbumin    | 2750                 | 1600^c             | 950                       |
| Ovomucoid     | 850                  | 937^d              | 213                       |
| Lysozyme      | 660                  | 441^e              | 220                       |
| L2            | 560                  |                    |                           |

^a Calculated from Fig. 5, using 346 daltons per nucleotide.
^b Based on the approximate polypeptide molecular weight (4) using Mw = 115 per amino acid.
^c Based on amino acid composition analysis which assumed a molecular weight of 78,700 (34).
^d Based on preliminary amino acid sequence data and a characterization of the in vitro translation product which contains a precursor with 23 additional amino acids (35).
^e Based on the known amino acid sequence (129 amino acids) and on an in vitro synthesized precursor with 18 additional amino acids (35).

Fig. 4. Determination of sufficient CH3HgOH concentration for electrophoresis under fully denaturing conditions. The 1.2% agarose tube gels containing increasing concentrations of CH3HgOH were prepared from stocks of 2.4% agarose in Buffer E and 50 mM CH3HgOH. Total poly(A)-containing RNA (1.5 µg) and 2 µg of E. coli rRNA was applied to each gel. Following electrophoresis, gels were stained and viewed as described under "Experimental Procedures." Apparent molecular weights were calculated from calibration lines determined by the E. coli rRNAs. Uw and Un are the relative mobilities of a given species in the presence and absence of CH3HgOH, respectively (21).

Effect of KCl Concentration — Another possible parameter affecting the synthesis of full length cDNA is KCl concentration. Synthesis in the absence of salt has been reported to increase the proportion of full length product using ovalbumin mRNA as template with AMvirus reverse transcriptase (39). We used each of the four mRNA fractions from Experiment 2 as templates for reverse transcriptase in reactions containing KCl at concentrations between 0 and 140 mM. With each template maximal incorporation was observed at 70 mM KCl (Fig. 9). The similar contour of the curves suggests that the effect of KCl concentration on cDNA yield is not template-specific. Poor incorporation into conalbumin cDNA correlates with the absence of full length conalbumin cDNA in Fig. 6b.

The length distributions of cDNAs synthesized with varying concentrations of KCl were determined on denaturing agarose gels. In the case of ovalbumin cDNA (Fig. 10a) the size distribution of each of these products is very similar. Each is composed of a major band migrating in the position expected for full length ovalbumin cDNA and a similar distribution of partial products. The same result was obtained with cDNA synthesized in 0 or 140 mM KCl using the other three mRNA fractions (Fig. 10b to d). We conclude that while KCl concentration does have an effect on the yield of cDNA, it has little influence on the product size distribution.
Con, ov, Mu, Lys +

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FIG. 6. Electrophoresis of total poly(A)-containing RNA and cDNA made from total poly(A)-containing RNA. In a, total poly(A)-containing RNA was electrophoresed on barbital-buffered formamide, 3.5% acrylamide gels as described under "Experimental Procedures." Gel 1 was loaded with 108 µg and Gel 2 with 42 µg plus 2 µg of E. coli rRNA. Both gels contain 2 µg of E. coli tRNA. The positions of 23 S and 16 S rRNAs on Gel 2 were determined by electrophoresis of these markers on a separate gel run in parallel (data not shown). The bands of ovalbumin (Ov) and conalbumin (Con) mRNAs and the approximate positions of ovomucoid (Mu) and lysozyme (Lys) mRNAs were determined from their migrations relative to E. coli rRNA. Gels were stained and viewed as described under "Experimental Procedures." In b, [32P]cDNA was synthesized from total poly(A)-containing RNA in a standard reaction using [32P]dGTP (16 cimol), electrophoresed on a 20 mM CH3HgOH-2% agarose slab gel and autoradiographed as described under "Experimental Procedures." DNA markers (not shown) were the nick-translated HindIII fragments of SV40 DNA. The arrow marks the position expected for full length conalbumin cDNA.

High concentrations of deoxynucleotide triphosphates have been reported to increase the average size of cDNA (39-43). In two of these reports full length cDNA could be obtained with low dXTP concentrations and the proportion of long cDNA increased by raising dXTP concentration (40, 41). In the other reports (39, 40, 43) only a small amount of the cDNA was long or full length at all dXTP concentrations. Where discussed (39, 41) investigators have found higher dXTP concentrations to enhance the yield of cDNA. We also observed this effect on total incorporation for reactions between 50 and 100 µM dXTPs with the four mRNA templates (Fig. 11). However, within the range of dXTP concentrations, no change in the size distribution of cDNA could be detected for any of the mRNA fractions (Fig. 12). Ovalbumin cDNA in each track of Fig. 12 was synthesized with a different concentration of dXTP present, using a constant specific radioactivity. cDNAs were synthesized from the other mRNA fractions (Fig. 12, b to d) with either 50 or 1000 µM dXTPs. While high dXTP concentrations increase the yield of cDNA, above 50 µM there is no substantial effect on product size distribution using otherwise standard conditions.

Comparison of Enzyme Preparations—All cDNA shown in Figs. 7 to 12 was prepared with AM virus reverse transcriptase, Lot G-1176 at 10 units/µg of RNA. We also tested a second enzyme preparation, Lot G-577 from the same source for its ability to catalyze the synthesis of full length ovalbumin cDNA. The relative yields and size distributions of ovalbumin cDNA made with varying amounts of the two enzyme lots is presented in Fig. 13. Up to 7 units/µg of mRNA, yields with the two lots are comparable. At enzyme:RNA ratios above 1

FIG. 7. The time course of ovalbumin cDNA synthesis at 37°, 42°, and 46°. Ovalbumin cDNA was synthesized under otherwise standard conditions at 37° (●), 42° (▲), and 46° (■) with [3H]dCTP (1.4 cimol). Points for all three curves represent separate 10-µl reactions prepared from a single mixture containing ovalbumin mRNA (500 µg/ml) at 10 µg/ml. Values represent the total trichloroacetic acid-precipitable incorporation and are the average of duplicate determinations.

FIG. 8. The effect of time and temperature on the size distribution of ovalbumin cDNA. Track 3 is ovalbumin cDNA synthesized under standard conditions with 10 µg/ml of ovalbumin mRNA and [32P]dGTP (1.5 cimol) and electrophoresed on a 20 mM CH3HgOH-2% agarose slab gel and autoradiographed as described under "Experimental Procedures." DNA markers (not shown) were the nick-translated HindIII fragments of SV40 DNA. The arrow marks the position expected for full length conalbumin cDNA.

Comparison of Enzyme Preparations—All cDNA shown in Figs. 7 to 12 was prepared with AM virus reverse transcriptase, Lot G-1176 at 10 units/µg of RNA. We also tested a second enzyme preparation, Lot G-577 from the same source for its ability to catalyze the synthesis of full length ovalbumin cDNA. The relative yields and size distributions of ovalbumin cDNA made with varying amounts of the two enzyme lots is presented in Fig. 13. Up to 7 units/µg of mRNA, yields with the two lots are comparable. At enzyme:RNA ratios above 1
FIG. 9. The effect of KC1 concentration on total incorporation into cDNA. cDNA was synthesized with either ovalbumin (Ov) (a), conalbumin (Con) (A), ovomucoid (Mu) (O), or lysozyme (Lys) (A) mRNA prepared in Experiment 2. Other than varying the KC1 concentration, standard reactions were done with 10 μg/ml of mRNAs, [3H]dCTP (0.8 Ci/mmol), and [3H]dATP (0.9 Ci/mmol). Values represent the total trichloroacetic acid-precipitable incorporation and are the average of two determinations. The background value of 1700 cpm for a no enzyme reaction has been subtracted from each point.

FIG. 10. The effect of KC1 concentration on the size distribution of cDNA. In a, ovalbumin cDNA was synthesized in reactions containing 2 μg/ml of ovalbumin mRNA, [32P]dGTP (6 Ci/mmol), and varying [KC1]: Track 1, 0 mM KC1; Track 2, 35 mM KC1; Track 3, 70 mM KC1; and Track 4, 140 mM KC1. In b, conalbumin cDNA was synthesized in reactions containing 5 μg/ml of conalbumin mRNA [32P]dGTP (18 Ci/mmol) and varying [KC1]: Track 1, 0 mM KC1, and Track 2, 140 mM KC1. In c, ovomucoid cDNA was synthesized in reactions containing 2 μg/ml of ovomucoid mRNA with [32P]dGTP (12 Ci/mmol); and in d, lysozyme cDNA was synthesized in reactions containing 2 μg/ml of lysozyme mRNA and [32P]dGTP (12 Ci/mmol). The [KC1] was varied for tracks in Panels c and d as in Panel b. All of the mRNAs were prepared in Experiment 2 and all other conditions for cDNA synthesis were standard. Within each panel an equal amount of radioactivity was applied per track. Electrophoresis was on 20 mM CH3COOH-agarose slab gels, using 1.5% agarose in Panels a and b and 2.0% agarose in Panels c and d. The nick-translated HindIII fragments of SV40 DNA were run in parallel slots on all gels (not shown). The slight curvature of tracks in Panel a was due to an edge artifact which was apparent in the migration of the dye front for the gel.
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phoria t3H\[rRNA (40,000 cpm/µg) to 50 ~1 of reverse transcriptase reaction mixtures containing either 3.5 or 60 units of one of the two enzyme preparations and incubating for 60 min at 42°. Following incubation, 1 ~1 was acid-precipitated and the remainder of each sample concentrated by ethanol precipitation and electrophoresed on 98% formamide, 3.2% acrylamide tube gels (23). After electrophoresis gels were sliced and counted (see "Experimental Procedures"). Although each sample contained the same amount of acid-precipitable radioactiv-

ity after incubation, the two samples incubated with enzyme from Lot G-577 displayed considerably more RNA degradation than samples incubated with equivalent units of enzyme from Lot G-1176. Hence, it seems probable that the two lots of reverse transcriptase vary in the proportion of full length synthesis due to different levels of contaminating ribonuclease activity.

**Determination of Molecular Weight and SI Nuclease Resistance** – In order to determine whether the major species of cDNA synthesized from each of the mRNAs was the full length copy, we determined their molecular weights on

| cDNA     | Length bases | Native | Denatured high salt assay | Denatured low salt assay |
|----------|--------------|--------|--------------------------|-------------------------|
| Ovalbumin| 1900 ± 100   | 83     | 7.6                      | 2.0                     |
| Conalbumin| 2500 ± 200  | 84     | 8.8                      | 2.1                     |
| Ovomucoid| 830 ± 50     | 81     | 7.1                      | 1.0                     |
| Lysozyme | 630 ± 50     | 81     | 7.9                      | 2.0                     |

**Table III**

Determination of cDNA lengths and SI nuclease resistances

The length of the major cDNA species synthesized from each mRNA fraction prepared in Experiment 2 was determined on CH3HgOH-agarose slab gels. Each value is the average of at least three determinations whose values varied less than 5%. The HindIII fragments of SV40 DNA served as molecular weight standards. cDNAs used to determine SI nuclease resistances were prepared from the various templates under standard conditions in reactions containing 0.3 µg of mRNA (3H)CTP (0.84 Ci/mm mol) and (3H)ATP (0.94 Ci/mm mol). Conditions used to determine the resistance of SI nuclease in high salt are stated under "Experimental Procedures." Low salt assays were carried out in an identical manner except that the NaCl concentration was 50 mM and only half as much SI nuclease was used. cDNAs were denatured by heating at 100° for 5 min in 20 µa NaCl.

**Fig. 11.** The effect of deoxynucleotide triphosphate concentration on total incorporation into cDNA. cDNA was synthesized with either ovalbumin (Ov) (●), conalbumin (Con) (○), ovomucoid (Mu) (●), or lysozyme (Lys) mRNA (△) preparations from Experiment 2. Other than varying the [dXTPs], reactions were done under standard conditions with 2 µg of mRNA/ml, [3H]CTP (0.84 Ci/mm mol), and [3H]ATP (0.94 Ci/mm mol). Values represent the total trichloroacetic acid-precipitable incorporation and are the average of two determinations.

**Fig. 12.** The effect of deoxynucleotide triphosphate concentration on the size distribution of cDNA. The amounts of mRNA per reaction, specific radioactivity and gel porosities are identical with the information given for the respective panels in Fig. 10. The [dXTP] for ovalbumin cDNA in Panel a were Track 1, 80 µM; Track 2, 150 µM; Track 3, 350 µM; Track 4, 500 µM; Track 5, 1000 µM. Conalbumin cDNA seen in Panel b was synthesized with either 50 µM dXTPs, Track 1, or 1000 µM dXTPs, Track 2. The [dXTPs] were varied for the two tracks of ovomucoid cDNA in Panel c and lysozyme cDNA in Panel d as in Panel b.
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Fig. 13. Titration of different reverse transcriptase lots with ovalbumin mRNA. Ovalbumin cDNA was synthesized in 10-μl standard reaction mixtures (25 μg/ml mRNA, 5 Ci/mmol of [3H]dGTP) with varying amounts of either reverse transcriptase Lot G-1176 (○) or Lot G-577 (●). Reactions were terminated with 100 μl of 10 mM dGTP containing 40 μg of E. coli tRNA. In Part a, the vertical axis represents the acid-precipitable radioactivity in a 5-μl aliquot (average of two determinations). Samples were prepared for electrophoresis and analyzed on alkaline, 2% agarose as described under "Experimental Procedures." In b, ovalbumin cDNA prepared with G-1176; in c, ovalbumin cDNA prepared with G-577. Tracks for both parts are: Track 1, 1 unit/μg of mRNA; Track 2, 3.5 units/μg of mRNA; Track 3, 7 units/μg of mRNA; Track 4, 25 units/μg of mRNA; and Track 5, 60 units/μg of mRNA.

CH₃HgOH-agarose slab gels. Figs. 10 and 12 are examples. The nick-translated HindIII fragments of SV40 DNA were run in adjacent slots and gave calibration curves in which migration was linear with the log molecular weight over the desired range. Table III gives the average of several molecular weight determinations for the major band in each cDNA. These lengths agree well with the expected values if cDNA synthesis is initiated near the 5' end of mRNA poly(A) tracts.

The S1 nuclease resistance of each cDNA was determined for the reverse transcriptase product before and after its denaturation at 100°C for 5 min in 20 mM NaCl (Table III). As previously observed for globin cDNA (41), the high S1 nuclease resistance (90 to 85%) of the native structure suggests that most cDNA remains bound to template after synthesis. The S1 nuclease resistance of denatured cDNA was determined at two NaCl concentrations, 300 and 50 mM. The S1 resistance after boiling dropped to less than 10%, consistent with the cDNA having been freed from template.

DISCUSSION

We have obtained conalbumin, ovalbumin, ovomucoid, and lysozyme mRNAs in a rapid, convenient fashion and found them to be suitable templates for reverse transcriptase. Although immunoprecipitation of specific polysomes (11) would probably yield mRNAs of greater purity, the predominance of these mRNAs in the oviduct and their differences in sedimentation rates have allowed us to obtain sufficient enrichments by size fractionation of total poly(A)-containing RNA on sucrose gradients (Fig. 1).

All four mRNAs are longer than necessary to code for the polypeptides (Table II). In the case of ovalbumin and conalbumin mRNAs there appear to be approximately 900 untranslated nucleotides. Inasmuch as the protein products synthesized in vitro are not noticeably greater in size than the authentic proteins, we cannot ascribe many extra nucleotides to large precursors, although a signal peptide (44) of approximately 20 to 30 amino acids might be undetected. Precursor sequences of about 20 amino acids were recently described for ovomucoid and lysozyme (35) and are taken into account in our estimate of untranslated nucleotides for the respective mRNAs. In each case some of the nucleotides, probably about 50, can be accounted for by a 3'-polyadenylic acid sequence.

Estimates of the molecular weight of ovalbumin mRNA have varied considerably (11, 38, 45). The difficulties in determining mRNA molecular weights by comparison to rRNA standards of defined size stem from differences in base composition and secondary structure not readily eliminated even in 98% formamide (37). The methylmercury concentration dependence of electrophoretic mobility (Fig. 4 and Ref. 21) is consistent with all RNAs tested having been fully denatured by 20 mM CH₃HgOH. Calculations of mRNA molecular weight from relative mobilities in this medium therefore appear to be valid.

Conalbumin mRNA is unusual in at least two respects. First, it is translated poorly in the rabbit reticulocyte lysate, whether using the intact system (12) or that rendered mRNA dependent by prior treatment with micrococcal nuclease. Second, conalbumin mRNA is a poor template for full length cDNA synthesis (Fig. 6). Data on total incorporation suggest that conalbumin mRNA is a relatively inefficient template for reverse transcriptase although uncertainty about the purity of the conalbumin mRNA fraction in Experiment 2 (Fig. 2) does not allow firm conclusions. One of the assumptions in defining abundance classes has been that all mRNAs have equal transcriptional efficiencies with reverse transcriptase (46, 47). This assumption may not be valid for oviduct mRNAs.

In investigating several of the parameters involved in cDNA synthesis we found the total incorporation to be influenced by the time of incubation, KCl concentration, and deoxynucleo-
tide triphosphate concentration. However, in contrast to a number of previous reports (39-43), none of these variables had an effect on the product size distribution over the ranges tested. A high proportion of cDNA was full length in each case. The latter two variables were examined with each of the four mRNA templates with the same result. Incubation temperature also does not substantially affect product length or the extent of total incorporation between 37° and 46°.

Two lots of reverse transcriptase were found to differ in their ability to catalyze the synthesis of full length ovalbumin cDNA (Fig. 13). Higher levels of ribonuclease were found in the less efficient enzyme lot in spite of its higher specific activity. Perhaps some of the conditions previously reported to increase the proportion of full length cDNA do not act on reverse transcriptase itself, but on contaminating activities instead.

In the accompanying paper, oviduct cDNAs are used as templates for E. coli DNA polymerase I. Second strand synthesis is investigated with the objective of providing high yields of double-stranded cDNAs which subsequently can be cloned in bacterial plasmids.

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