Synthesis of a Deoxyribonucleic Acid Sequence Complementary to Ovalbumin Messenger Ribonucleic Acid and Quantification of Ovalbumin Genes*

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SUMMARY

DNA was synthesized in vitro from a template of purified ovalbumin messenger RNA. The DNA product is complementary to a homogeneous sequence specific for ovalbumin. The amount of ovalbumin sequence in hen oviduct and hen liver DNA was measured by hybridization experiments and found to be 2 copies per diploid genome in both tissues, indicating that there is no selective amplification of ovalbumin genes in the tissue specialized for ovalbumin synthesis.

Both DNA (1-3) and RNA (4) polymerases can be used to synthesize nucleic acid sequences complementary to hemoglobin messenger RNA. Such sequences, specific for a given polypeptide, can be used in molecular hybridization experiments to estimate the quantity of information coding for that polypeptide in a given population of nucleic acid molecules (5-7).

We have previously reported the purification of messenger RNA for chicken ovalbumin by selective immunoadsorption of ovalbumin polyribosomes and subsequent adsorption of messenger RNA to nitrocellulose filters (8). The present communication describes the preparation of DNA complementary to this messenger RNA using the RNA-directed DNA polymerase of Rous sarcoma virus (9). Analysis of this DNA by molecular hybridization experiments indicates that the enzymatic product represents a homogeneous set of nucleotide sequences specific for ovalbumin, and that 2 copies of these sequences per diploid cell are present in both hen oviduct and hen liver. We conclude that the extensive synthesis of ovalbumin by oviduct cells cannot be attributed to gene amplification.

MATERIALS AND METHODS

Most of the materials used have been described elsewhere (8). [3H]dATP (17.6 Ci per mmole), [3H]dCTP (30 Ci per mmole), [3H]dTTP (13.5 Ci per mmole), and [3H]thymidine (40 Ci per mmole) were obtained from Schwarz-Mann, [3H]dGTP (9.8 Ci per mmole) from Amersham, oligo(dT) (14 to 16 nucleotides in length) from Collaborative Research, and hydroxyapatite (Bio-Gel HTP, "DNA grade") from Bio-Rad; actinomycin D was a generous gift from Merck, Sharp and Dohme. Antiovalbumin antibody, specific for ovalbumin and free of RNase, and matrices of cross-linked ovalbumin were prepared as described previously (8).

Preparation of Purified Ovalbumin mRNA—Details of this procedure and controls on the homogeneity of the product have been described at length elsewhere (8). Further documentation of its homogeneity is provided below (see "Results"). Polysomes were isolated from adult hen oviduct by centrifugation to a sucrose cushion (10). These polysomes (10 A260 units per ml) in polysome buffer (0.025 M NaCl-0.005 M MgCl2-0.025 M Tris-HCl, pH 7.6, containing 100 μg of heparin per ml) were incubated with purified goat antiovalbumin antibody (0.5 mg per ml) for 45 min at 4°C. A matrix of cross-linked ovalbumin (200 mg of matrix per ml of reaction mixture) was then added to the reaction mixture, and the mixture was incubated for 45 min more at 4°C with constant stirring. The mixture was then centrifuged at 4°C for 10 min at 6,000 rpm in the Sorvall HB4 rotor, the supernatant fluid was discarded, and the matrix was washed three times with 0.5 M sucrose, 0.15 M NaCl, 1% Triton X-100, and 1% sodium deoxycholate in polysome buffer (1 ml of buffer per 100 mg, wet weight, of matrix), then one time with polysome buffer alone. To elute the immunoadsorbed polysomes, a buffer containing 0.01 M Tris-HCl, 0.05 M EDTA, pH 7.5, and 100 μg of heparin per ml was added (0.5 ml/100 mg, wet weight, of matrix) and incubated for 15 min at 4°C with constant stirring. The preparation was centrifuged as before and the supernatant fluid was saved. Two more elutions were performed and the

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three supernatant fluids were pooled. The pooled supernatant fractions were made 0.1 M in NaCl and precipitated with 2 volumes of ethanol at -20° for 12 hours. The precipitate was then dissolved in 0.5% SDS-0.02 M sodium acetate 0.005 M EDTA, pH 5.0, at a final concentration of 10 A_{260} per ml. One-milliliter samples were layered on 11.5 ml 5 to 20% sucrose gradients in the same buffer and centrifuged at 40,000 rpm for 6 hours at 20° in a Spinco SW 41 rotor. The gradient was pumped through a flow cell in a Gilford recording spectrophotometer, and all material sedimenting more rapidly than the tRNA and SDS-treated proteins was collected and precipitated with ethanol three times as described above. This RNA was then adsorbed to Millipore filters according to a modification (8) of the procedure of Drawerman et al. (11) to selectively enrich for mRNA.

We have previously documented the efficacy of our procedure for preparing ovalbumin-specific polyribosomes (8, 10, 12, 13). The messenger RNA obtained from these polyribosomes is almost entirely specific (95%) for ovalbumin (8). Our procedure does not eliminate all ribosomal RNA, but this fact does not appreciably complicate the use of the messenger RNA as a template for DNA synthesis (see below).

Preparation of DNA from Chicken Tissue—DNA was prepared by homogenizing chicken oviduc or liver tissue in 0.1 M NaCl, 0.05 M Tris-HCl, pH 8.2, and 0.01 M EDTA with six strokes in a Dounce homogenizer. SDS was added to 0.5% and the material was homogenized with three more strokes. Pronase (self-digested in 0.02 M Tris 0.01 M EDTA, pH 7, for 2 hours at 37°) was added to a final concentration of 200 µg per ml and the preparation was incubated overnight at 37°. More SDS was added to a final concentration of 1%, the material was extracted twice with an equal volume of phenol (previously saturated with 0.02 M sodium acetate), and 2 volumes of ethanol were added. After 48 hours at -20° the material was centrifuged for 20 min at 10,000 rpm in a Sorvall H44 rotor, and the precipitate was dissolved in 0.001 M Tris-HCl 0.01 M EDTA, pH 7.0. Ribo-nuclease (Wothington, DNAase-free, previously boiled for 10 min at 10 mg per ml) was added to a final concentration of 100 µg per ml and the preparation was incubated overnight at 37°. More SDS was added to a 10-fold excess over the amount required to obtain complete hydrolysis of the susceptible substrate. At the end of the incubation period 40 µg of native calf thymus DNA were added as carrier, the samples were precipitated with trichloroacetic acid and filtered, and the amount of acid-precipitable radioactivity was measured. For further details of this procedure, see Leong et al. (16). The quantity of nuclease-resistant material is expressed as a percentage of the acid-precipitable radioactivity in the samples incubated without S1 nuclease.

Hydroxyapatite Fractionation of Nucleic Acids—Samples were diluted into several milliliters of 0.1 M sodium phosphate, pH 6.8, adsorbed to hydroxyapatite at room temperature, and eluted at 60° first with 0.16 M then with 0.4 M sodium phosphate, pH 6.8, using a previously described batch procedure (16). The material eluted by 0.16 M sodium phosphate was taken to be single stranded, that eluted by 0.4 M sodium phosphate to be double stranded. Single and double stranded standards (boiled phage λ DNA and native SV40 DNA, respectively) both fractionated as approximately 95% pure under these conditions.

In Vitro DNA Synthesis with RSV DNA Polymerase—The method of purification of RSV DNA polymerase from Rous sarcoma virus and the conditions of DNA synthesis were as previously described (9), except that the phosphocellulose column step was omitted in the purification of the polymerase. Single stranded DNA was synthesized with a mixture containing 0.1 M Tris-HCl, pH 8.1, 0.008 M MgCl₂, 0.3 M β-mercaptoethanol, 100 µg of actinomycin D per ml (17), 0.1 µg of oligo(dT) per ml, 7 × 10⁻³ M dATP, 7 × 10⁻³ M TTP, 1.6 × 10⁻³ M [³H]dGTP (0.8 Ci per mmole), 1.6 × 10⁻³ M [³H]dCTP (30 Ci per mmole), 1.3 µg of the ovalbumin messenger RNA per ml, and 0.2 enzyme units of purified RSV DNA polymerase per ml. Labeled nucleoside triphosphates were evaporated to approximately 0.1 volume to remove ethanol before being added to the reaction mix. This mixture was incubated for 16 to 18 hours at 37°, then SDS was added to 0.5% and the mixture was incubated at 55° for 5 min more. HeLa cell RNA, 20 µg prepared as described previously (9), was added as carrier along with sodium acetate to 0.2 M and ethanol to 67% (v/v). The sample was allowed to precipitate at -20° for at least 6 hours. After centrifugation at 14,000 × g for 20 min, the precipitate was dissolved in 200 µl of 0.4 M EDTA, boiled RNAase was added to 100 µg per ml, and the sample was incubated for 1 hour at 37° to destroy the template RNA and disrupt DNA-RNA hybrids (16). At the end of this time the sample was diluted into 2 ml of 0.01 M sodium phosphate, pH 6.8, and fractionated on hydroxyapatite as described above. The single stranded material, about 70% of the total DNA synthesized, was then passed through a column (40 × 0.9 cm) of Sephadex G 50 (coarse) in 0.3 M NaCl, 0.01 M Tris HCl, pH 8.1, 0.001 M EDTA, and 0.1% SDS. The void volume was collected, ethanol was added to 67% (v/v), and the sample was precipitated at -20°.

Double stranded DNA was synthesized under identical conditions except that actinomycin D was omitted from the enzymatic reaction. After elution from hydroxyapatite, the fraction eluting in 0.4 M sodium phosphate was passed through the Sephadex column. This double stranded material was found to contain a fraction (about 40% of the total) resistant to denaturation. This resistant material was removed by boiling the double stranded DNA for 10 min in 0.003 M EDTA, pH 7, adsorbing the DNA to hydroxyapatite again, and eluting the single stranded material as described above.
The amount of DNA synthesized in vitro was never sufficient for optical measurement of the mass of DNA present. From the specific activities of the nucleoside triphosphates used in the synthesis of this DNA, and from our estimates of the base content of the DNA product (Table II, below), we estimate the specific activity of the DNA product to be about 8000 cpm per µg.

**Assay of Ovalbumin mRNA Activity in Cell-Free Protein-synthesizing System**—This technique, consisting of immunoprecipitation of ovalbumin synthesized in a rabbit reticulocYTE lysate system, was as described previously (18) except that isoleucine was used as the labeled amino acid (8).

**Isolation of Hen Oviduct Monosomal RNA**—Hen oviduct monosomes were purified by rate zonal centrifugation in a 0.5 to 1.5 M sucrose gradient (19) and deproteinized by centrifugation in an SDS-sucrose gradient as described previously (8). This RNA was assayed in the reticulocYTE lysate protein synthesis system (see above) and found to be 3% as active as ovalbumin synthesis per unit mass of RNA as hen oviducT polysomal RNA, and about 0.1% as active as our purified messenger RNA (data not shown).

**DNA-DNA Hybridization**—DNA samples were denatured by boiling for 10 min in 0.003 M EDTA, pH 7. Salt was then added was used as the labeled amino acid (8).

**Preparation of Labeled, Unique Sequence Chicken DNA**—Chick embryo fibroblasts were cultured for 48 hours in the presence of 2.5 µCi of [3H]thymidine per ml. DNA was then prepared by adsorption and elution from hydroxyapatite essentially as described by Britten et al. (22), except that the lysed cells were sheared by passage through a 26-gauge needle. The double stranded DNA was then sheared at 50,000 p.s.i as described above and dialyzed against 0.003 M EDTA. This material was precipitated by the addition of ethanol, then denatured and reannealed as described above to a Cdt value of 6 x 10^8 mole·s per liter. The material was fractionated by hydroxyapatite (see above), and the single stranded material was dialyzed against 0.005 M EDTA and precipitated by addition of ethanol. This material was considered to be unique sequence chicken DNA; it had a specific activity of 10,000 cpm per µg.

**RESULTS**

**Template Specificity of RSV DNA Polymerase and Characterization of DNA Product**—Purified ovalbumin mRNA supports DNA synthesis at a rate even greater than that obtained with RSV 70 S RNA (Table I). In a 50-µl reaction mixture containing 65 ng of purified ovalbumin mRNA, 80,000 cpm or about 10 ng of DNA were synthesized. Preincubating the purified ovalbumin mRNA with RNase destroyed the template activity, indicating that RNA, not a DNA contaminant, was directing the synthesis. The requirement for oligo(dT) is in agreement with the findings of others for globin mRNA (1-3) and presents the reciprocal of the percentage of the probe that is double stranded when that population of molecules has reacted for an infinite time (21). Since RNA was present in great excess the concentrations of the RNA species determined the kinetics of the reaction, and the homogeneity and complexity measured by these reactions are those of the RNA, not the DNA species. This type of analysis of RNA-DNA hybridization reactions is discussed more fully by Birnstiel et al. (21).

**TABLE I**

| Additions to polymerase system | Acid-precipitable H | cpm/50-µl reaction |
|------------------------------|---------------------|--------------------|
| Purified ovalbumin mRNA (1.3 µg per ml) | 79,000 | 79,000 |
| Purified ovalbumin mRNA (1.3 µg per ml) and RNase | 250 | 250 |
| Polymerase system alone (no added RNA) | 950 | 950 |
| Polymerase system alone plus RNase | 200 | 200 |
| Purified ovalbumin mRNA (1.3 µg per ml), no oligo(dT) | 900 | 900 |
| RSV 70 S RNA (2 µg per ml) | 51,000 | 51,000 |
| RSV 70 S RNA (2 µg per ml) and RNase | 380 | 380 |
| Monosomal RNA (1.2 µg per ml) | 2,000 | 2,000 |
| Monosomal RNA (1.2 µg per ml) and RNase | 300 | 300 |
| Monosomal RNA (1.2 µg per ml) and purified ovalbumin mRNA (1.3 µg per ml) | 84,000 | 84,000 |

Additions to polymerase system: 1 µl of each reaction mixture contained 50 pg of DNA, 0.1 ng of purified ovalbumin mRNA, 200 units of RSV 70 S RNA polymerase, 50 units of pancreatic RNase if any, was preincubated for 30 min at 37° with 100 µg of boiled pancreatic RNase per ml before this mixture was added to the polynucleotide reaction mixture. The reaction conditions were as described under "Materials and Methods," except that at the end of the synthesis reaction the material was precipitated by the addition of acid and radioactivity was counted as described previously (17).
ovalbumin mRNA and monosomal RNA produced approximately the same amount of synthesis as purified mRNA alone, indicating 3% of the amount of DNA synthesis in the presence of purified ovalbumin mRNA. A reaction mixture containing both purified synthesis (including endogenous background synthesis) was only that the low synthesis in the presence of monosomal RNA was "Materials and Methods" and Ref. 8). The amount of DNA synthesis of that in the reaction with added RNA, no steps were taken to further purify the enzyme, although such techniques have been developed (9).

We have previously presented evidence (8) that our purified ovalbumin mRNA still contains rRNA. Although other studies (1, 9) have shown that mRNA is not transcribed by DNA polymerase from tumor viruses, we tested whether hen mRNA might serve as template under our conditions of synthesis. RNA from hen oviduct monosomes was isolated by sucrose gradient centrifugation (see "Materials and Methods") and tested for relative ability to direct DNA synthesis in vitro (Table I). This monosomal RNA had a higher relative concentration of mRNA than the purified ovalbumin mRNA but much less messenger RNA (see "Materials and Methods" and Ref. 8). The amount of DNA synthesis (including endogenous background synthesis) was only 3% of the amount of DNA synthesis in the presence of purified ovalbumin mRNA. A reaction mixture containing both purified ovalbumin mRNA and monosomal RNA produced approximately the same amount of synthesis as purified mRNA alone, indicating that the low synthesis in the presence of monosomal RNA was not the result of an inhibitor in this fraction. From these experiments we conclude that less than 3% of the DNA synthesis in the presence of purified ovalbumin mRNA is directed by ribosomal or viral RNAs.

The single stranded enzymatic product has a range of sedimentation coefficients centering about 7 S (Fig. 1). This indicates an average chain length of about 200 nucleotides, and compares favorably with the size of DNA transcribed from larger RNA templates under similar conditions (see "Discussion").

The nucleotide composition of the enzymatic product was determined with DNA synthesized in the presence of all four radioactive nucleoside triphosphates (Table II). The base ratios of ovalbumin mRNA are not known; therefore we cannot state whether the nucleotide composition of the DNA product is appropriate for a sequence complementary to this mRNA. However, the proportion of thymidine is high, perhaps indicating the presence of some poly(T) sequences in the product complementary to the poly(A) sequences in the mRNA. Such poly(T) sequences would presumably interfere with the specificity of the DNA product. To prevent these putative poly(A)-poly(T) sequences from being scored in assays for secondary structure, the DNA product in all subsequent experiments was synthesized with deoxycytidine and deoxyguanosine as the only labeled nucleotides, and X1 nuclease was used to analyze the secondary structure of nucleic acids.

To show that these precautions are sufficient to prevent poly(A)-poly(T) duplexes from being scored, the experiment shown in Table III was performed. Incubation of the DNA product with poly(A) to a very large C1t value did not produce an appreciable amount of labeled double helical structures.

**Specificity of DNA Product for Ovalbumin Sequences.** To investigate the nature of that RNA in the messenger fraction used as template by the RSV polymerase, single stranded DNA

| Nucleotide | Amount | Specific activity | Ratio, cpm: specific activity | Relative percentage |
|-----------|--------|------------------|-----------------------------|-------------------|
| dC        | 3,500  | 30               | 117                         | 8                 |
| dA        | 5,800  | 17.6             | 331                         | 22                |
| dG        | 2,000  | 6.8              | 203                         | 14                |
| T         | 11,000 | 13.5             | 806                         | 55                |

**Base ratios of DNA product**
Hybridization of DNA product with poly(A)

[3H]DNA product at 1 ng per ml was annealed for 1 hour either to itself or to poly(A) at 50 μg per ml to a Cₜ of 1 mole·s per liter relative to the poly(A). [3H]Poly(T) (see "Materials and Methods") was annealed to a 1000-fold excess of poly(A) to a Cₜ value of 1 mole·s per liter. The samples were then assayed for secondary structure by S1 nuclease. Conditions of RNA-DNA hybridization and nuclease digestion were as described under "Materials and Methods."

| Nucleic acid                        | Nuclease resistance after annealing |
|-------------------------------------|------------------------------------|
| [3H]DNA product                    | 6                                  |
| [3H]DNA product plus poly(A)        | 8                                  |
| [3H]Poly(T) plus poly(A)            | 100                                |

Tissue specificity of RNA complementary to DNA product

RNA was extracted from purified Rous sarcoma virus, chicken fibroblasts, or hen tissue by phenol at 60°C, pH 9.0. The RNA was then treated with DNase (Worthington, RNase-free), extracted against with phenol at 25°C, pH 9.0, and precipitated with ethanol. For further details of this procedure see Leong et al. (16). Ovalbumin mRNA was prepared as described under "Materials and Methods." Conditions of hybridization of these RNAs with the [3H]DNA product and assay of secondary structure by S1 nuclease were described under "Materials and Methods." The Cₜ (2 × 10⁻³ mole·s per liter) achieved in the experiment with RSV RNA is sufficient to make >90% of the DNA complementary to RSV RNA double stranded (17).

| RNA added                          | Cₜ relative to RNA added | Incubation time | Nuclease resistance |
|------------------------------------|--------------------------|-----------------|---------------------|
| None                               | 0                        | 0 s             | 4                   |
| None                               | 18                       | 6 s             | 3                   |
| RSV RNA                            | 2 × 10⁻¹                  | 2               | 7                   |
| Chicken fibroblast RNA (cells not transformed) | 10⁻¹                     | 20              | 9                   |
| Chicken fibroblast RNA (cells transformed with RSV) | 5 × 10⁻¹                  | 20              | 3                   |
| Total hen liver RNA                | 4 × 10⁻¹                  | 20              | 7                   |
| Total hen oviduct RNA             | 10⁻¹                     | 20              | 101                 |
| Ovalbumin mRNA preparation         | 10⁻¹                     | 18              | 93                  |

The DNA product was hybridized to different RNA preparations (Table IV).

The DNA product does not hybridize with itself, with the HeLa RNA present in all RNA-DNA hybridization reactions (see "Materials and Methods"), with RNA from a tissue which does not synthesize ovalbumin, or with 70 S RSV RNA. By contrast, the product does hybridize almost completely with both total oviduct RNA and purified ovalbumin mRNA. The kinetics of hybridization of either purified ovalbumin mRNA or total oviduct RNA with the DNA product are illustrated in Fig. 2. The reciprocal of the percentage nuclease resistance was plotted as a function of the reciprocal of the Cₜ (see "Materials and Methods"). For both ovalbumin mRNA and total oviduct RNA, the data (Fig. 2) fit a single straight line intersecting the ordinate near the point 0.01. We conclude (see "Materials and Methods") that nearly 100% (1/0.01) of the DNA product is complementary to one homogeneous RNA species found in both total hen oviduct RNA and our purified mRNA. The alternate interpretation, that the DNA product is complementary to multiple species of RNA, would require that each of those RNA species be present at nearly identical concentrations (see "Discussion").

The Cₜ₁₉ for the hybridization reaction with purified ovalbumin mRNA is 3.8 × 10⁻³ mole·s per liter. The Cₜ₄₁₉₂ for the reaction of poliovirus RNA with single stranded DNA made from poliovirus RNA template is 4.7 × 10⁻³ mole·s per liter. The polio genome is unique, with a complexity⁴ of 7500 nucleotides (28). By comparison of the Cₜ₄₁₉₂ of the messenger RNA fraction with that of the poliovirus RNA, the apparent complexity of our preparation of ovalbumin mRNA is 6000 nucleotides. This complexity is consistent with the DNA product being comple-

⁴ Complexity is the amount of nucleic acid, measured in daltons or in nucleotides, necessary to code for the repetitive unit of information in a population of nucleic acid molecules.
Fig. 3. Comparison of relative protein synthesizing ability and rate of hybridization of RNAs. Chicks given secondary stimulation with estrogen (10) were killed 18 hours later, and polysomes were isolated from their oviducts (see "Materials and Methods"). Previous experiments (8) have indicated that approximately 15% of the ovalbumin messenger from chicks in this state of secondary stimulation are specific for ovalbumin. An aliquot of these polysomes was separated by the ovalbumin-antiovalbumin system into an immunoadsorbed and a nonimmunoadsorbed fraction, and the RNA was then isolated from these polysomes as described under "Materials and Methods." This RNA was not fractionated by the Millipore filter method. RNA from total oviduct polysomes (C), immunoadsorbed polysomes (O), or nonimmunoadsorbed polysomes (Δ) was assayed at different concentrations in the reticulocyte lysate system (see "Materials and Methods") for the ability to synthesize ovalbumin (A). Aliquots of the same RNAs were tested for rate of hybridization with [3H]DNA product (B). The hybridization reaction had 5 ng of [3H]DNA product per ml, and either 27 µg (for total oviduct polysomal RNA, □), 4 µg (for immunoadsorbed polysomal RNA, ○), or 64 µg (for nonimmunoadsorbed polysomal RNA, Δ) of polysomal RNA per ml. Conditions of RNA-DNA hybridization and of the S1 nuclease assay are described under "Materials and Methods." The C, values are based on the concentration of polysomal RNA present. In the table (B, inset) ovalbumin synthesizing ability per unit mass of RNA and the reciprocal of the C, of each of these three RNAs are compared. Values are expressed relative to the value determined for total oviduct polysomes.

To gain further information on the RNA complementary to the DNA product, the experiment described in Fig. 3 was performed. Polysomes from immature chicks that had received estrogen stimulation were fractionated by immunoadsorption into populations containing different proportions of ovalbumin messenger. Nor do these results exclude the possibility that the probe is complementary to a RNA composed of 1500 nucleotides and comprising approximately 25% of the RNA in the messenger preparation. Both values are near our estimates for the size and relative purity of our ovalbumin mRNA (8). However, this experiment does not prove that the RNA is ovalbumin messenger. Nor do these results exclude the possibility that the probe is complementary to a contaminating RNA species of low complexity and present as only a few per cent of the purified mRNA preparation.

Quantification of Ovalbumin DNA Sequences in Chicken Genome—To measure the absolute number of copies of the sequence coding for ovalbumin in the chicken genome, the following experiment was performed. A large amount of either chicken liver or chicken oviduct DNA was denatured and reassocaited in the presence of [3H]DNA product specific for ovalbumin and a trace amount of 14C-labeled unique sequence chicken DNA. If the ratio of sequences in the unlabeled cellular DNA to identical sequences in the labeled DNA is high, the rate of reassociation of the cellular ovalbumin sequences will be determined by the concentration of identical unlabeled cellular sequences. Therefore by comparing the C, of the labeled, ovalbumin-specific product to that of the labeled unique sequence DNA, the relative reiteration of these two species in the genome can be determined. From the data presented in Fig. 4 it is clear that for both liver DNA (Panel A) and oviduct DNA (Panel B) the C, of the ovalbumin sequences is not significantly different from that of the unique sequences. From these results we conclude that both

6 To determine the effect of the added DNA product on the rate of reassociation of the cellular ovalbumin sequences the following
FIG. 4. Determination of the absolute copy number of ovalbumin sequences in the chicken genome. 3H-labeled single stranded DNA product at 1 ng per ml, 14C-labeled unique sequence chicken DNA at 300 ng per ml (both prepared as described under "Materials and Methods"), and unlabeled cellular DNA from chicken liver, chicken oviduct, or calf thymus (see "Materials and Methods") at 10 mg per ml were mixed together, then denatured and reannealed as described under "Materials and Methods." At different times aliquots were taken and assayed for resistance to S1 nuclease (see "Materials and Methods"). The data are plotted relative to the Cot of the unlabeled cellular DNA.

A, 14C-labeled unique sequence DNA (O) and [3H]ovalbumin specific DNA product ( ), reassociated with chicken liver DNA. B, 14C-labeled unique sequence DNA (O) and [3H]ovalbumin specific DNA product ( ), reassociated with chicken oviduct DNA; [3H]unique sequence DNA ( ) and [3H]ovalbumin specific DNA ( ), reassociated with calf thymus DNA.

calculations were performed. A = number of copies present (in both cellular DNA and DNA product) of the single stranded sequence identical with the DNA product. B = number of copies present of the single stranded sequence complementary to the DNA product. S = number of single stranded copies of DNA product present. D = number of double stranded, cellular DNA copies of the ovalbumin sequence present.

\[
A = D + S \\
B = D
\]

The single stranded DNA product and the double stranded, cellular DNA were melted and mixed together (Fig. 4). All DNA of the same sequence should now react at the same rate, regardless of whether it originally was from the DNA product or the cellular DNA. Since 70% of the labeled DNA reassocated at the maximum Cot value tested:

\[
0.7 (A) = B \\
0.7 (D + S) = D \\
D = 2.33 S
\]

There are a minimum of 2.3 copies of the ovalbumin sequence present in the cellular DNA per unit mass of DNA. If the reaction proceeds further at greater Cot values, this ratio will be even greater. The maximum possible effect of the DNA product on the Cot/2 of the cellular DNA, and on our estimate of the number of ovalbumin sequences per diploid genome, is to increase it by a factor of \((1 + 2.3)/2.3 = 1.4\).
After reassociation the DNA was fractionated on hydroxyapatite to select the double stranded molecules (see "Materials and Methods") and these molecules were then denatured and assayed for secondary structure (Fig. 6). In order to have a control of perfectly paired DNA duplexes specific for ovalbumin, double stranded DNA product was reassociated only with itself, then denatured and assayed under identical conditions. We conclude that a very high percentage of the nucleotides in the cellular DNA-DNA product duplex are correctly base paired.

**DISCUSSION**

Analysis of the data presented in this paper depends on the homogeneity and specificity of the RNA template used in the synthesis of the DNA product. We have previously presented evidence concerning the purity of our ovalbumin mRNA (8, 10). In brief, ovalbumin polysomes are selectively isolated from the total oviduct polysomes by immunoadsorption with antiovalbumin antibody and an ovalbumin matrix (see "Materials and Methods"). A variety of indirect controls with polysomes from other tissues, with other antibodies and with other matrices, indicated that both the binding of antibody to the polysomes and the subsequent binding of the polysome-antibody complex to the matrix have a high degree of specificity (8, 10). In more direct controls, the pattern of nascent peptide chains in the immunoadsorbed polysomes was studied and found to be consistent with that expected for pure ovalbumin polysomes (8). Furthermore, immunopurification of polysomes from the ovoduct of either estrogen-stimulated immature chicks (at the time when ovalbumin comprised about 17% of the total protein being synthesized) or of mature hens (where ovalbumin is about 65% of the total protein synthesized) resulted in increases of 7-fold and 1.5-fold, respectively, in the amount of ovalbumin synthesized in vitro per unit mass of RNA (8). All of these experiments indicated that more than 90% of the mRNA in our purified ovalbumin mRNA preparation is specific for ovalbumin. However, a large fraction (about two-thirds) of the RNA in this mRNA preparation is still composed of rRNA (8).

DNA synthesis is dependent on the addition of an RNA species in the ovalbumin mRNA preparation. This RNA species is not rRNA (see Table I and "Results"), nor is it tRNA, since tRNA, by virtue of its size, is separated from the ovalbumin mRNA by rate zonal sedimentation (see "Materials and Methods" and Ref. 8). The dependence of the DNA synthesis reaction on oligo(dT) strongly implies the presence of a poly(A) region in the template RNA molecules. Finally, the amount of DNA product synthesized per unit mass of RNA template is fairly large. Addition of 65 ng of purified RNA to the DNA synthesis reaction resulted in synthesis of about 10 ng of DNA product ("Results" and Table I). Since a large proportion of the added RNA template was rRNA (8), which is not transcribed by this polymerase (Ref. 16 and Table I), and since the tumor virus RNA-dependent DNA polymerases have never been found to synthesize a significant excess of DNA product relative to the amount of template present (1, 9, 30, 31), the DNA product must be synthesized from a large proportion of the non-ribosomal RNA sequences in the mRNA preparation, not just from a minor contaminant. Since a large proportion of the mRNA molecules are transcribed, and since ovalbumin mRNA comprises almost all the mRNA molecules present, we believe the majority of the DNA sequences are complementary to ovalbumin mRNA. More direct evidence supporting this conclusion comes from the fact that the complementary RNA sequence is specific to the chicken oviduct (Table IV), and that the complementary RNA...

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**Fig. 6.** Denaturation of DNA-DNA duplexes. $^3$H-Labeled double stranded DNA product (at 5 ng per ml) was annealed either by itself (to a $C_0^+$ value of $10^{-6}$ mole $\cdot$ per liter relative to the $[^3]$H$\cdot$DNA product) or in the presence of 5 mg of hen liver DNA per ml (to a $C_0^+$ value of $10^{-4}$ mole $\cdot$ per liter relative to the $[^3]$H$\cdot$DNA product) under the conditions described under "Materials and Methods" for DNA-DNA hybridization. The reassociated portion of the DNA was then isolated by adsorption to and elution from hydroxyapatite (see "Materials and Methods") and passed through a column (40 X 0.9 cm) of Sephadex G-50 (coarse) in 0.1 M NaCl, 0.01 M Tris-HCl, pH 8.1, 0.001 M EDTA, and 0.1% SDS; the void volume was collected and precipitated at $-20^\circ$ with 2 volumes of ethanol. The samples were then dissolved in 0.01 M Tris- HC1, pH 8.1, and aliquots were incubated at the indicated temperatures for 15 min, then immediately quenched at $0^\circ$. These aliquots were then assayed for secondary structure by the S1 nuclease assay (see "Materials and Methods"). $^3$H labeled double stranded DNA product, reassociated with itself alone, O---O; $^3$H-labeled double stranded DNA product, reassociated in the presence of chicken liver DNA, $\bullet-\bullet-\bullet$.

Duplexes under identical conditions and comparing the results. The amount of cellular DNA present in the reassociation reaction was enough to decrease the $C_0^+$ at which the material is 40% renatured by a factor of 2.5 (Fig. 5). Therefore 60% of the $[^3]$H$\cdot$DNA that has reassociated is now paired with liver DNA.

Since the rate of reassociation of the [H]DNA product is increased by a factor of 2.5 by the amount of cellular DNA added, the concentration of ovalbumin sequences is 2.5 times greater with the addition of the cellular DNA than without it. That means there are 1.5 cellular DNA copies of the ovalbumin sequence for every 1 copy in the [H]DNA product. The ratios of the various possible combinations of labeled and unlabeled duplexes are

|        |        |
|--------|--------|
| + strand | - strand |
| Labeled | Labeled | 0.4 - 0.4 = 0.16 |
| Labeled | Unlabeled | 0.4 - 0.4 = 0.24 |
| Unlabeled | Labeled | 0.6 - 0.4 = 0.24 |
| Unlabeled | Unlabeled | 0.6 - 0.6 = 0.36 |

Therefore 16% of the duplexes contain 2 labeled strands, and 48% contain 1 labeled strand. However, the duplexes in which both strands are labeled have twice the specific activity of the molecules in which only one strand is labeled. After reassociation, when these double stranded molecules are isolated on hydroxyapatite, 60% of the radioactivity is in molecules in which 1 strand is [H].DNA product and 1 is unlabeled cellular DNA.
is purified from other oviduct RNAs by immunoadsorption in an ovalbumin-anti-ovalbumin system to the same degree that ovalbumin mRNA is purified (Fig. 3).

The size of the DNA product (7 S, Fig. 1) is considerably smaller than the proposed size of ovalbumin mRNA (about 16 S, Ref. 18). The largest product, 8 S, of RNA-directed DNA polymerase reported to date was obtained with a relatively small template, globin mRNA, 10 S (1, 2). DNA synthesized with larger templates, such as 70 S viral RNA (32), 35 S poliovirus RNA (15), and mRNA for mouse k chain immunoglobulin (30), have all been approximately the same size as the product reported here. However, the short chain length of the DNA does not preclude extensive or complete representation of template nucleotide sequences in the enzymatic product (33).

In Fig. 2 the data for hybridization of DNA product with ovalbumin mRNA fit well to a straight line. We conclude that almost all of the DNA product is complementary to one homogeneous RNA species. The alternate interpretation, that the DNA product is complementary to multiple species all of which hybridize at the same rate with complementary sequences in the DNA product, seems unlikely, since it would require each of those multiple RNA sequences to be present at the identical molar concentration, each to be specific to the oviduct (Table IV), and each to be purified to the same degree by immunoadsorption (Fig. 2).

The presence of minor contaminating DNA species in the DNA product, complementary to sequences other than that of ovalbumin mRNA, would not invalidate the conclusions drawn from the experiments presented here since the experiments are based on the kinetics of hybridization of the major kinetic component of the DNA product.

We do not have a sufficient amount of the DNA product to measure its concentration optically. We can only estimate the concentration of DNA product from the amount of radioactivity present and the specific activity of the DNA. Therefore we can neither get a reliable estimate of the complexity of the double stranded DNA, nor can we calculate the absolute number of copies of the ovalbumin gene from Fig. 5.

Ovalbumin comprises approximately 60 to 65% of the soluble protein synthesized in the oviduct (10), yet the number of ovalbumin sequences seems to be 1 per haploid chicken genome, and seems not to be amplified in tissue specialized for ovalbumin synthesis. Similar evidence exists for a lack of gene amplification in hemoglobin-specific DNA sequences in duck (5, 7) and mouse globin sequences seems to be 1 per haploid chicken genome, and which are not translated by ribosomes. The stringency of this assay, can detect perhaps 3% mismatching by a 2^0 decrease in Tm (36). This hybridization assay should prove a useful method for quantitation of ovalbumin sequences in different physiological or developmental states and should be useful in analysis of the structure of the genome in higher organisms.

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