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Ovalbumin Messenger RNA: Evidence That the Initial Product of Transcription Is the Same Size as Polysomal Ovalbumin Messenger

(complementary DNA/hybridization/RNA processing/oviduct)

G. STANLEY McKNIGHT* AND ROBERT T. SCHIMKE†

Department of Biological Sciences, Stanford University, Stanford, California 94305

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ABSTRACT **The messenger RNA for ovalbumin, the major secretory protein of the chick oviduct, appears not to be made as a high-molecular-weight precursor when artifacts due to aggregation are eliminated. No ovalbumin messenger RNA sequences that will hybridize to complementary DNA made against ovalbumin mRNA are found in concentrated samples of hen oviduct RNA larger than 28 S. The sensitivity of the hybridization assay is sufficient to detect less than one molecule of ovalbumin mRNA precursor per tubular gland cell. Newly synthesized ovalbumin messenger RNA isolated from immature chicks stimulated briefly by estrogen is the same size as that found in hen polyribosomes. We conclude that ovalbumin messenger RNA does not undergo any significant change in molecular weight from its initial transcription to its incorporation into polyribosomes.**

The synthesis of messenger RNA in animal cells and its transport from the nucleus to the cytoplasm has received much attention in recent years, but our understanding of this process is incomplete and confused. A model for mRNA synthesis has been proposed by Darnell and coworkers (1) which involves the initial synthesis of a large precursor RNA with subsequent post-transcriptional addition of poly(A) sequences to the 3′ terminus. This large precursor is degraded except for the 3′ portion containing the mRNA, which is then transported to the cytoplasm and incorporated into polysomes.

A large amount of evidence supporting this hypothesis has been reported. Both heterogeneous nuclear RNA (HnRNA) and mRNA contain a 3′-terminal poly(A) region of approximately the same length (2). Jelinek *et al.* (3) have reported that much of the poly(A) synthesized in the nucleus is conserved and transported to the cytoplasm. However, Perry and coworkers (4) have recently suggested that most of the nuclear poly(A) remains in the nucleus and is degraded there. Further evidence for the precursor-product relationship between HnRNA and mRNA has been obtained for specific mRNAs by either translation of the HnRNA *in vitro* (5, 6) or by its hybridization to complementary DNA (7). Such results are not completely convincing because the possibility of aggregation artifacts in sucrose gradients has not been rigorously excluded. Proof that a large precursor exists for a specific mRNA should involve separation of the precursor from polysomal mRNA and a careful demonstration of its covalent integrity by a variety of denaturing techniques. This has not been reported for any proposed precursor, and in cases where dimethylsulfoxide (Me_2SO) gradients have been used, most of the large mRNA sequences dissaggregate to polysomal mRNA sizes (7). In this study we report evidence suggesting that the mRNA coding for chick oviduct ovalbumin (OV) is synthesized, transported to the cytoplasm, and incorporated into polysomes without a measurable change in its molecular weight.

Abbreviations: OV, ovalbumin; HnRNA, heterogeneous nuclear RNA; $NaDodSO_4$, sodium dodecyl sulfate; Me_2SO, dimethylsulfoxide.

* Present address: Faculté de médecine, Strasbourg, France.
† Reprint requests should be sent to Dr. Robert T. Schimke, Department of Biological Sciences, Stanford University, Stanford, Calif. 94305.

MATERIALS AND METHODS

Chicks, Injections, and RNA Labeling. The magnum portion of the oviduct from either laying hens or chicks was used in this study. Four-day-old, female White Leghorn chicks were given 10 days of primary stimulation with estradiol benzoate (1 mg/day) and then withdrawn from the hormone for 6–8 weeks as described (8). Secondary stimulation was initiated by intramuscular injection of 3 mg of estradiol benzoate to the withdrawn chicks. RNA was labeled with [^{3}H]uridine by explanting small fragments of hen magnum in Hank's salts solution containing 0.35 g/liter of sodium bicarbonate, 25 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.0), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 500 μCi/ml of [5-^{3}H]uridine (27 Ci/mmol) for 15–30 min at 37°.

RNA Isolation. Nuclei were not used because of possible RNA degradation during their isolation. Total RNA was extracted from oviduct magnum by Dounce homogenization in 9 volumes (w/v) of TNM buffer [10 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, 25 mM NaCl] containing 5% (w/v) sucrose, 1 mg/ml of sodium heparin, 1% Triton X-100, and 1% sodium deoxycholate at 4°. An equal volume of 2× sodium dodecyl sulfate ($NaDodSO_4$) buffer [1% $NaDodSO_4$, 10 mM Tris·HCl (pH 7.5), 5 mM EDTA] was added, and the entire mixture was extracted with an equal volume of phenol–chloroform (1:1) by shaking 30 min at 25° and centrifugation at 15,000 × *g* for 15 min to separate the phases. The aqueous phase was re-extracted twice with phenol–chloroform and made 0.2 M in NaCl. The nucleic acid was precipitated with 2.5 volumes of ethanol at −20° overnight. RNA was freed of contaminating phenol–chloroform by repeated ethanol precipitations or by washing the precipitated RNA with 70% ethanol containing 0.1 M NaCl. DNA was removed by two to three washes of the ethanol precipitate with 3 M sodium acetate, 5 mM EDTA as described (9) and subsequent reprecipitation with ethanol to remove the salt. DNase digestions where indicated were performed in TNM buffer for 1 hr at 25° with 20 μg/ml of DNase (Worthington RNase-free) that had been treated with iodoacetate to remove small amounts of contaminating RNase (10). DNase was removed

by phenol–chloroform extraction. Polysomal RNA was isolated as described (11).

RNA Sedimentation in Sucrose Gradients. RNA samples were lyophilized to remove ethanol, dissolved in 0.5 ml of $NaDodSO_4$ buffer, heat-treated at 65° for 10 min, and cooled rapidly in an ice–salt bath. The RNA was layered directly on either 15–30% or 5–20% (w/v) linear sucrose gradients made in $NaDodSO_4$ buffer and centrifuged in an SW41 rotor at 41,000 rpm, 25° in a Beckman L-350 centrifuge for the times indicated. Fractions (0.5–1 ml) were collected from the bottom of the tube. Palmiter (12) demonstrated that the 65° heat treatment method disaggregated OVmRNA without reducing its ability to direct OV synthesis in a reticulocyte lysate. Other workers (13, 14) have also found similar heat treatments to effectively disaggregate RNA without causing thermal degradation.

Isolation of >28S RNA. Total RNA was centrifuged on 5–20% sucrose–$NaDodSO_4$ gradients for 5 hr. The top part of the gradient was removed leaving the bottom 2.5 ml, which contained RNA sedimenting at about 28 S or larger. This RNA was then concentrated by ethanol precipitation. Using pulse-labeled RNA, we find no loss of RNA using this procedure. Large RNA concentrates in the bottom milliliters of the gradient but does not form a firm pellet because of its high solubility.

RNA·DNA Hybridization. Complementary DNA (cDNA) was synthesized from purified OVmRNA with RNA-dependent DNA polymerase from Rous sarcoma virus as described (15) with the following modifications. The cDNA was labeled only with [^{3}H]dCTP (30 Ci/mmol) and was treated with 0.3 M NaOH for 16 hr to destroy RNase and contaminating RNA. The specificity of this cDNA for OVmRNA sequences has been rigorously demonstrated (15). Hybridizations were performed at 68° in 0.3 M NaCl, 10 mM Tris·HCl (pH 7), 2 mM EDTA, in a total volume of 20 μl. Reticulocyte RNA (0.5 mg/ml) and 0.1% $NaDodSO_4$ were added to inhibit any RNase present. Hybrid formation was assayed by diluting the reactions with 1 ml of S1 buffer [30 mM Na acetate (pH 4.5), 3 mM $ZnCl_2$, 0.3 M NaCl, 10 μg/ml of denatured and 10 μg/ml of native calf thymus DNA]. S1 single-strand specific nuclease is a significant contaminant in α-amylase (Sigma Grade IV) from *Aspergillus oryzae* and was obtained by dissolving α-amylase in S1 buffer at 4 mg/ml. The enzyme (400 μg) was added to each reaction mixture, which was then incubated at 37° for 2 hr. Sigma α-amylase is also slightly contaminated with a double-stranded nuclease activity, but does not digest RNA·DNA hybrids under these conditions (McKnight, unpublished data). Trichloroacetic acid-precipitable radioactivity was measured (15) and expressed as percent of control incubations of cDNA without S1 digestion. Three to 6% of the cDNA was nuclease resistant in the absence of OVmRNA, and these values were subtracted. The specific activity of the cDNA is about 8000 cpm/ng, and 175–200 cpm were used in each hybridization.

RESULTS

Recovery of RNA. We examined the recovery of both nuclear RNA and OVmRNA in our isolation procedures because of the possibility of selective loss of mRNA into the phenol phase (16) or loss of nuclear RNA in the protein interface (17). Total RNA was extracted from hen tissue pulse-labeled for 15 min with [^{3}H]uridine. Much of the label at this time is larger than 28 S, and more than 80% of the radioactivity is confined to the nucleus. The recovery of RNA, measured by A_{260}, was 65%; the recovery of radioactivity was 70%, measured by comparing the RNA in the first ethanol precipitate to that found in the original homogenate. Since approximately 10% of the aqueous phase is lost in each of the three extractions used to separate RNA from protein, the corrected yield would be close to 100%. OVmRNA was measured by either its biological activity in a reticulocyte lysate or by its hybridization to cDNA. The amount of OVmRNA found per gram of oviduct by this phenol extraction method is as much as we have found by any other total or polysomal RNA isolation method (18).

RNA Aggregation. One of the major problems we encountered in attempting to demonstrate the existence of a large OVmRNA precursor was aggregation‡. After phenol extraction of total RNA we have found that a variable amount, and often as much as 50%, of the OVmRNA can sediment faster than 18 S on sucrose–$NaDodSO_4$ gradients when no attempt is made to denature the RNA. The rapidly sedimenting forms of OVmRNA have discreet sizes (23 S and 35–40 S), can be separated from each other, and do not change their sedimentation behavior when rerun under nondenaturing conditions. We have used several methods to denature these rapidly sedimenting OVmRNA sequences with variable success. Treating the RNA with 85% Me_2SO at 37°, followed by ethanol precipitation as suggested by Katz and Penman (19), caused some changes in sedimentation behavior, but large amounts of OVmRNA still sedimented faster than 18 S. Sedimentation of total RNA on sucrose gradients made in 99% Me_2SO does not completely disrupt large aggregates of 18 S OVmRNA unless great care is taken in sample preparation. Our most successful results with Me_2SO gradients were obtained by the method of Firtel (20) and included a 65° heat treatment before the sample was applied.

Aggregation of OVmRNA sequences is completely eliminated on aqueous $NaDodSO_4$–sucrose gradients by use of the 65° heat treatment described in *Materials and Methods*, and we therefore examined the effects of this treatment on high-molecular-weight RNA preparations. High-molecular-weight RNA (>28 S) was isolated from total RNA that had not been heat-treated and mixed with a small amount of 30–50S HeLa-[^{3}H]RNA. Half of the sample was heat-treated. The sedimentation of the chicken RNA (Fig. 1a) and HeLa RNA (Fig. 1b) with and without heat treatment is shown. The heat treatment disaggregated most of the chicken RNA to 28 S and 18 S. After heat treatment some of the HeLa RNA melted to 45 S, but no increase in RNA sedimenting slower than 30 S is seen. The sedimentation of the heat-treated HeLa RNA appears similar to the sedimentation behavior of this RNA in 99% Me_2SO gradients. Fig. 1c demonstrates that pulse-labeled >28S hen oviduct RNA can be isolated and concentrated by the techniques described in *Materials and Methods*. DNase digestion and repeated heat treatments have no effect on the pattern of this high-molecular-weight RNA as shown.

Conversion of Percent Hybridization to Nanograms of OVmRNA. The hybridization results shown in Figs. 3 and 4 are

‡ OVmRNA sediments at 18 S on sucrose gradients made in 99% Me_2SO. However, on sucrose–$NaDodSO_4$ aqueous gradients, OVmRNA actually sediments at 16 S, perhaps because of different conformations. We will refer to unaggregated OVmRNA throughout this paper as 18 S for simplicity.

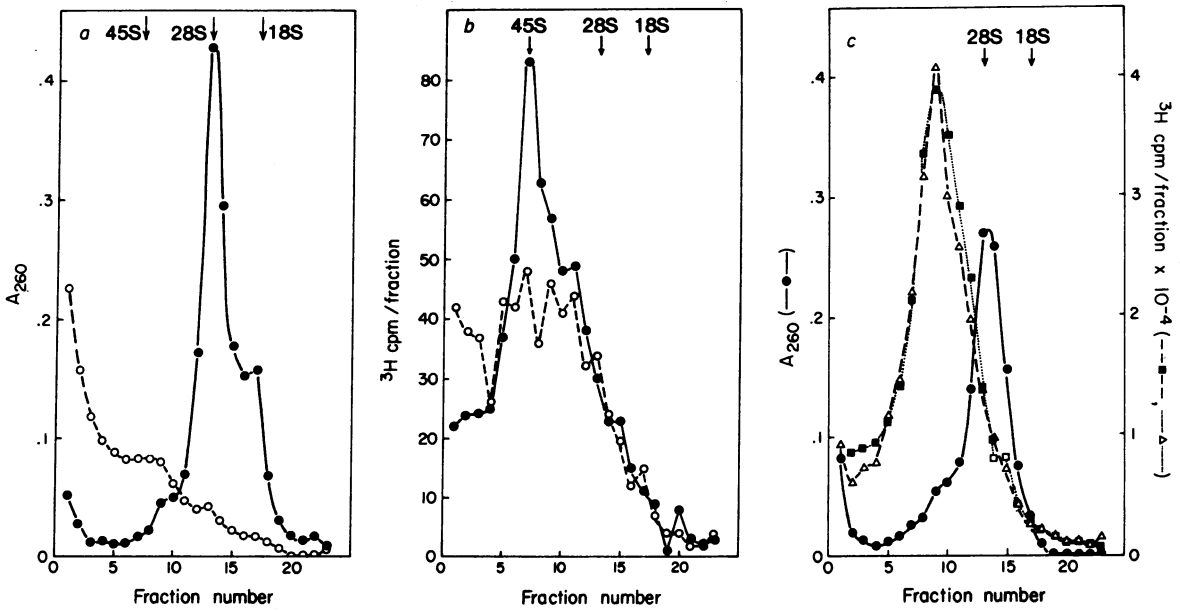

FIG. 1. Effects of heat treatment on high-molecular-weight RNA. Total hen oviduct RNA, isolated as described in *Materials and Methods*, was centrifuged on 5–20% sucrose–NaDodSO$_4$ gradients without heat treatment, and the RNA larger than 28 S was collected. This >28S RNA was then mixed with 30–50S HeLa [^{3}H]RNA isolated by a NaDodSO$_4$ method (18) that does not cause extensive aggregation. Half of the RNA was heat-treated at 65° and both samples were then layered on linear 15–30% gradients and centrifuged for 5 hr. The absorbance profile of the hen RNA before (○) and after (●) heat treatment is shown in panel a. In panel b, the sedimentation of the 30–50S HeLa RNA is shown before (○) and after (●) heat treatment. In panel c, the >28S RNA from hen oviduct labeled for 30 min *in vitro* with [^{3}H]uridine was isolated as described in *Materials and Methods* and centrifuged on a 15–30% gradient for 5 hr (△). The same RNA was then treated with DNase and heat-treated again before centrifugation (■). Fractions (0.5 ml each) were collected on all gradients.

expressed as nanograms of OVmRNA in each gradient fraction. In order to convert percent hybridization to nanograms of OVmRNA, we performed the following experiment. A constant amount of cDNA was hybridized to varying amounts of pure OVmRNA for 24 hr, as described in *Materials and Methods*. The results shown in Fig. 2 give a standard curve relating percent hybridization to ng of OVmRNA. The samples in the two crucial experiments (Fig. 3c and Fig. 4a) were also hybridized for 24 hr under exactly the same conditions as in Fig. 2, using all the RNA in each gradient fraction. Therefore, for these two experiments percent hybridization was converted to nanograms of OVmRNA directly, using the curve in Fig. 2 without further corrections.

Fig. 2 indicates that values for percent hybridization over 60% cannot be accurately converted to nanograms of OVmRNA since there is very little change in percent hybridization between 0.4 and 20 ng of OVmRNA. To prevent the hybridization reactions from going over 60% in the experiments shown in Figs. 3a, 3b, and 4b, where there are large amounts of OVmRNA, we have hybridized cDNA to a small sample of each gradient fraction for times less than the standard time of 24 hr (details are in figure legends). The peak of OVmRNA in these gradients represents 50–60% hybridization of the cDNA. Percent hybridization was converted to nanograms of OVmRNA by first using Fig. 2 as described above; values were then corrected for both the time of hybridization and the amount of each fraction hybridized. For example, if we used 1% of a gradient fraction, hybridized for 1 hr, and found 55% hybridization, we would use Fig. 2 to convert 55% hybridization to 0.2 ng of OVmRNA, multiply by 100 (correction for using 1% of gradient fraction), and multiply by 24 (standard time/1 hr) to obtain a value of 480 ng or OVmRNA.

Hybridization of >28S RNA to cDNA. We examined the distribution of OVmRNA sequences in total cellular RNA from hen oviduct by sedimentation on gradients and hybridization to cDNA as described in *Materials and Methods*. Fig. 3a shows that no OVmRNA sequences larger than 18 S are detectable in total hen RNA. In the experiment shown in Fig. 3b, we concentrated the >28S RNA from hen RNA and find that most of the remaining OVmRNA sequences are still 18 S. We further concentrated and purified the >28S RNA by collecting the heavy fractions from Fig. 3b; the results are shown in Fig. 3c. There is now a well-defined peak of A_{260} at about 40 S, which we assume is partly ribosomal RNA precursor. Hybridization reveals no OVmRNA sequences. The

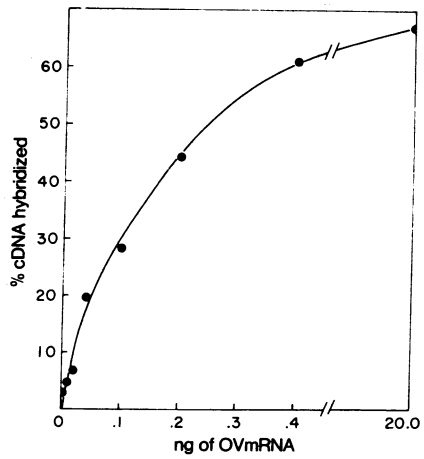

FIG. 2. Hybridization of pure OVmRNA to cDNA. Increasing amounts of pure OVmRNA (gift of D. Shapiro) were each hybridized to 175 cpm of cDNA for 24 hr in 20-μl reaction mixtures as described in *Materials and Methods*. In control tubes without OVmRNA, 6% of the cDNA became S1-resistant, and this blank has been subtracted.

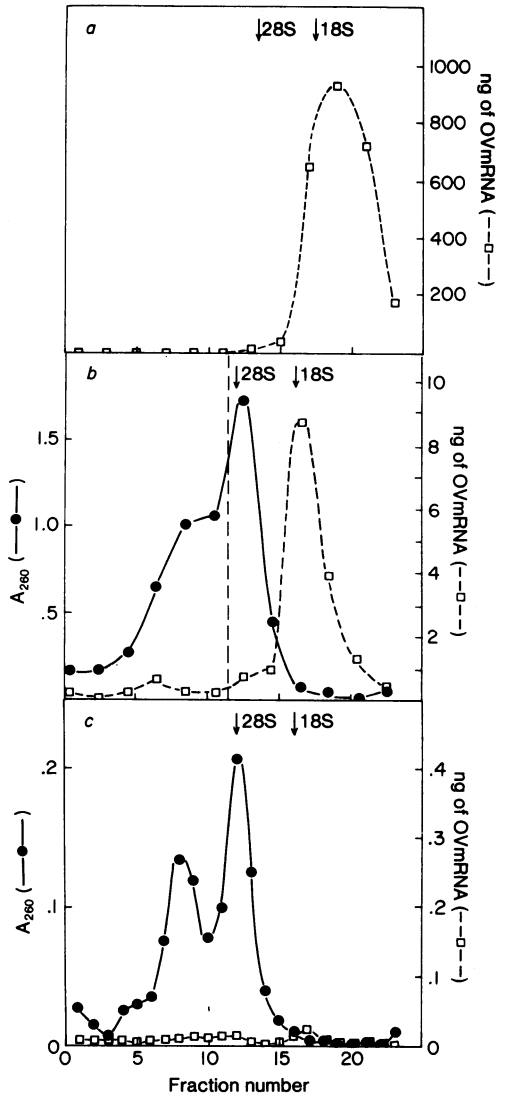

FIG. 3. (a) Hybridization of cDNA to hen total RNA. Four A_{260} units of hen total RNA were sedimented on a 15–30% gradient for 4 hr, as described in *Materials and Methods*. Fractions (1 ml each) were collected, and 10-μl samples of each fraction were hybridized to cDNA for 1 hr. (b) Hybridization of cDNA to a concentrated sample of >28S hen RNA. >28S hen RNA (6 A_{260} units) was sedimented on a 15–30% gradient for 5 hr. Fractions (1 ml each) were collected and 1/5 of the RNA in each fraction was hybridized to cDNA for 5.5 hr. (c) Hybridization to >28S hen RNA concentrated from 70 A_{260} units of hen total RNA. The RNA in panel b sedimenting faster than the RNA represented by the dashed line was concentrated and treated with DNase; 0.6 A_{260} unit (equivalent to the >28S RNA concentrated from 70 A_{260} units of total RNA) of it was recentrifuged on a 15–30% gradient for 5 hr. Fractions (0.5 ml) were collected. After A_{260} was determined, the total RNA in each fraction was precipitated with ethanol and hybridized to cDNA for 24 hr.

RNA in Fig. 3c represents the >28S RNA from 70 A_{260} units of hen RNA, which is equivalent to the RNA in 0.3 g wet weight of oviduct. From the number of tubular gland cells per g (7.1×10^8) (21) and the molecular weight of OVmRNA (700,000) (Shapiro, in preparation), we can estimate the expected amount of OVmRNA precursor in this RNA to be 0.23 ng if there were only one OVmRNA precursor per cell. Since no significant amount of OVmRNA larger than 28 S is seen,

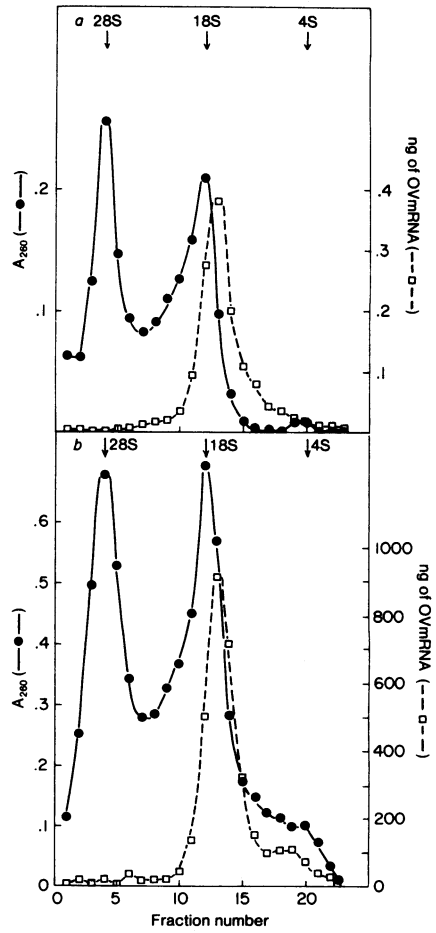

FIG. 4. (a) Hybridization to newly synthesized OVmRNA. 1.2 A_{260} units of total RNA from chicks given a secondary stimulation with estrogen for 4.5 hr was centrifuged on a 5–20% gradient for 6 hr. Fractions (0.5 ml) were collected and A_{260} was measured. After ethanol precipitation the total RNA in each fraction was hybridized to cDNA for 24 hr. (b) Distribution of OVmRNA in hen polysomal RNA. 3.2 A_{260} units of hen polysomal RNA were sedimented as described above. Two percent of the RNA in each fraction was hybridized to cDNA for 20 min.

we conclude that there is less than 1 molecule of OVmRNA precursor per cell.

Size Distribution of Newly Synthesized OVmRNA. The possibility that the precursor to polysomal ovalbumin mRNA is only slightly larger than 18 S could not be easily investigated using hen tissue because of the enormous concentration of polysomal OVmRNA in the cell which would mask a small pool of precursor. However, in the immature chick it is possible to achieve a state where OVmRNA synthesis is rapid but where little accumulation of polysomal mRNA has yet occurred. This developmental stage conforms to the ideal situation suggested by Darnell (1) for the detection of an HnRNA precursor to messenger. Four-day-old chicks were injected with estrogen for 10 days and then withdrawn from the hormone for 8 weeks, at which time ovalbumin synthesis is undetectable (McKnight, in preparation). A secondary injection of estrogen is then given, which after a lag period of 3 hr causes a large increase in OVmRNA as assayed by lysate activity (21), by hybridization to cDNA, or by measurement of the rate of ovalbumin synthesis (McKnight, in preparation). Fig. 4a shows the results of hybridization to total cellular

RNA isolated from secondary stimulated chicks that have been injected with estrogen for 4.5 hr. We find no OVmRNA sequences sedimenting faster than 18 S. Fig. 4b is a gradient of hen polysomal RNA run under similar conditions for comparison. At 4.5 hr after estrogen injection, OVmRNA synthesis has only been increased for 1.5 hr because of the 3-hr lag period. If the half-life of the precursor were 10 min or longer, as has been suggested for precursor mRNA in other systems (22), we calculate that the precursor to OVmRNA should account for at least 16% of the total OVmRNA in the cell under these conditions§. Precursor molecules larger than 28 S would have appeared in the bottom fraction since RNA is too soluble to pellet under these conditions. Since the size of all of the OVmRNA sequences at 4.5 hr is exactly the same as that found on hen polysomes, we conclude that any processing of OVmRNA that occurs after transcription either does not involve an appreciable change in molecular weight or occurs extremely rapidly.

DISCUSSION

We have not been able to find any evidence for the existence of a high-molecular-weight precursor to OVmRNA when aggregation of OVmRNA is eliminated. We have isolated the >28S RNA from sufficient cells to allow the detection of one molecule of precursor per cell by hydridization to cDNA. Even under these conditions, no OVmRNA sequences larger than 28 S were seen. Palmiter (21) has calculated, from the steady-state level of OVmRNA and its half-life, that the rate of synthesis of OVmRNA is approximately 30 molecules/min per cell. If only one molecule of precursor existed per cell, its half-life would have to be on the order of 1–2 sec, which is much shorter than the time necessary for transcription of a >28S molecule. Since the RNA isolation techniques used in this study allow the isolation of biologically active OVmRNA in high yields as well as the recovery of >28S RNA by both radioactivity (Fig. 1c) and absorbance (Fig. 3c), we conclude that degradation could not account for our inability to find a precursor.

In order to look for a precursor that might be only slightly larger than 18 S OVmRNA, we have taken advantage of the fact that OVmRNA levels are regulated by estrogen. A secondary administration of estrogen to withdrawn chicks causes a dramatic increase in the amount of OVmRNA per cell. During the early hours of secondary stimulation the rate of OVmRNA synthesis is rapid (21), but little polysomal OVmRNA has accumulated in the cytoplasm. Since a short-lived nuclear precursor should reach its steady-state level rapidly, the ideal time to detect it would be shortly after the increase in OVmRNA synthesis occurs. We have been able to find no evidence for OVmRNA sequences in any molecules larger than 18 S after 1.5 hr of estrogen-induced stimulation of OVmRNA synthesis (Fig. 4a). A precursor with a half-life as short as 10 min would have been clearly seen under these conditions.

Although the existence of high-molecular-weight HnRNA in animal cells has been well documented (14), we agree with the conclusion of Davidson and Britten (23) that a functional role has not been demonstrated. The experiments presented here strongly suggest that at least one mRNA is not made as a large precursor that must be cleaved to yield functional mRNA. We conclude that the hypothesis that HnRNA is a precursor to mRNA may not be true in all cases and that other possible functions for HnRNA such as a direct role in either gene regulation or chromosome structure should be considered.

§ The rate of accumulation of a molecule that is turning over is given by:

$$\frac{dX}{dt} = K_s - KdX$$

At the steady state, where $dX/dt = 0$ then $X = K_s/K_d$, where X is the molecule (units), K_s is a zero-order rate constant of synthesis (units/time), and K_d is a first-order rate constant of degradation (1/time). Assuming that OVmRNA synthesis commences and persists at a constant rate for the 90-min period between 3 and 4.5 hr after estrogen administration to chicks, and there is no degradation of total OVmRNA sequences, then

$$\text{total OVmRNA} = K_s \times 90 \text{ min.}$$

Assuming a 10-min half-life for an OVmRNA precursor, then the steady-state level of precursor (SS) is:

$$SS = K_s/K_d \text{ or } (K_s \cdot t_{1/2})/\ln 2$$

Therefore, the percent of OVmRNA sequences existing as precursor is:

$$(K_s 10/\ln 2)/K_s 90 \times 100 \text{ or } 16\%.$$

1. Darnell, J. E., Jelinek, W. R. & Molloy, G. R. (1973) *Science* **181**, 1215–1221.
2. Molloy, G. R. & Darnell, J. E. (1973) *Biochemistry* **12**, 2324–2329.
3. Jelinek, W., Adesnik, M., Salditt, M., Sheiness, D., Wall, R., Molloy, G., Philipson, L. & Darnell, J. E. (1973) *J. Mol. Biol.* **75**, 515–532.
4. Perry, R. P., Kelley, D. F. & LaTorre, J. (1974) *J. Mol. Biol.* **82**, 315–331.
5. Rruiz-Carrillo, A., Beato, M., Schultz, G., Feigelson, P. & Allfrey, V. G. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3641–3645.
6. Williamson, R., Drewienkiewitz, C. E. & Paul, J. (1973) *Nature New Biol.* **241**, 66–68.
7. Imaizumi, T., Diggelman, H. & Scherrer, K. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1122–1126.
8. Palmiter, R. D., Christensen, A. K. & Schimke, R. T. (1970) *J. Biol. Chem.* **245**, 833–845.
9. Palmiter, R. D. (1973) *J. Biol. Chem.* **248**, 2095–2106.
10. Zimmerman, S. B. & Sandeen, G. (1966) *Anal. Biochem.* **14**, 269–277.
11. Palacios, R., Palmiter, R. D. & Schimke, R. T. (1972) *J. Biol. Chem.* **247**, 2316–2321.
12. Haines, M. E., Carey, N. H. & Palmiter, R. D. (1974) *Eur. J. Biochem.* **43**, 549–560.
13. Ojala, D. & Attardi, G. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 563–567.
14. Holmes, D. S. & Bonner, J. (1973) *Biochemistry* **12**, 2330–2338.
15. Sullivan, D., Palacios, R., Stavnezer, J., Taylor, J. M., Faras, A., Kiely, M., Summers, M., Bishop, J. M. & Schimke, R. T. (1973) *J. Biol. Chem.* **248**, 7530–7539.
16. Palacios, R., Sullivan, D., Summers, M., Kiely, M. & Schimke, R. T. (1973) *J. Biol. Chem.* **248**, 540–548.
17. Penman, S. (1969) in *Fundamental Techniques in Virology* (Academic Press, New York), pp. 35–48.
18. Rhoads, R. E., McKnight, G. S. & Schimke, R. T. (1973) *J. Biol. Chem.* **248**, 2031–2039.
19. Katz, L. & Penman, S. (1966) *Biochem. Biophys. Res. Commun.* **23**, 557–560.
20. Firtel, R. A. & Lodish, H. F. (1973) *J. Mol. Biol.* **79**, 295–314.
21. Palmiter, R. D. (1973) *J. Biol. Chem.* **248**, 8260–8270.
22. Scherrer, K. (1973) in *Karolinska Symposia on Research Methods in Reproductive Endocrinology*, 6th Symposium, ed. Diczfalusy, E. (Karolinska Institute, Stockholm) p. 95.
23. Davidson, E. H. & Britten, R. J. (1973) *Quart. Rev. Biol.* **48**, 565–613.