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Processing of High Molecular Weight Ovalbumin and Ovomucoid Precursor RNAs to Messenger RNA

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Summary

The existence of poly(A) sequences in the multiple high molecular weight forms of ovalbumin and ovomucoid nuclear RNA has been determined. The results indicate that all the bands observed in the total nuclear RNA including the largest (7.8 kb) were also detected in the poly(A) RNA. Kinetic labeling and chase experiments in oviduct tissue-suspension system indicated that the ovalbumin and ovomucoid high molecular weight RNAs which were labeled with a short-time incubation can be chased by cold nucleosides and actinomycin D into mature mRNAs. The largest RNAs labeled in this oviduct suspension system have a size of 7.8 kb for ovalbumin and 5.5 kb for ovomucoid, which correspond respectively to the "a" bands of steady state RNA. The processing of the precursors to mature mRNA was also examined by electron microscopic analysis, by hybridizing oviduct nuclear RNA to probes which were isolated from different segments of intervening DNA sequences and by measuring the turnover kinetics of specific intervening sequences present in cellular poly(A) RNA. The results indicate that the seven intervening sequence regions of the ovomucoid precursor are removed in a preferred but not necessarily an obligatory order.

Introduction

Recent progress in the technology of gene cloning makes it possible to study the structural organization of genes. These studies have revealed that most eucaryotic structural genes (exons) coding for mRNA sequences are interrupted by intervening sequences (introns) (Brack and Tonegawa, 1977; Jeffreys and Flavell, 1977; Berget, Moore and Sharp, 1977; Chow et al., 1977; Tilgham et al., 1977; Dugaiczyk et al., 1978; Garapin et al., 1978; Lai et al., 1978; Mandel et al., 1978; Woo et al., 1978; Catterall et al., 1979; Dugaiczyk et al., 1979; Gannon et al., 1979). This surprising sequence organization of eucaryotic genes has prompted several groups to study how mRNAs are transcribed and processed from their natural genes (Ross, 1976; Curtis and Weissman, 1976; Bastos and Aviv, 1977; Kwan, Wood and Lingrel, 1977; Nevins and Darnell, 1978; Ross and Kneckt, 1978; Kinniburgh, Mertz and Ross, 1978; Schibler, Marcu and Perry, 1978; Tilghman et al., 1978; Kinniburgh and Ross, 1979). The accumulated evidence suggests that entire natural genes are transcribed as one large RNA precursor and then processed to mature messenger RNA. For example, a 15S RNA precursor to globin mRNA has been shown to be a transcript of the entire sequence of the globin gene (Tilghman et al., 1978). This precursor RNA must then be processed to mature mRNA by removing the intervening RNA sequences. In the oviduct system the natural ovalbumin gene has been demonstrated in our laboratory and others to contain seven intervening DNA sequences (Dugaiczyk et al., 1978, 1979; Garapin et al., 1978; Lai et al., 1978; Mandel et al., 1978; Woo et al., 1978; Gannon et al., 1979). In addition we have recently reported that the ovomucoid gene, which also codes for an egg white protein and is regulated by steroid hormones, is also interrupted by seven intervening sequences (Catterall et al., 1979; Lai et al., 1979).

In searching for the primary transcripts of these genes we have used the RNA blotting technique of Alwine, Kemp and Stark (1977) to screen oviduct nuclear RNA for ovalbumin and ovomucoid RNA sequences (Roop et al., 1978; Nordstrom et al., 1979). As reported earlier, multiple ovalbumin as well as ovomucoid RNA species larger than their mature mRNAs exist in oviduct nuclei. These findings are consistent with the hypothesis that ovalbumin and ovomucoid mRNAs are derived from larger RNA precursors. To substantiate this hypothesis we have carried out pulse labeling experiments to demonstrate that the high molecular weight RNAs which exist in oviduct nuclei are indeed the precursors to the mature mRNAs. In addition, the high molecular weight ovomucoid RNAs were analyzed by hybridization mapping in an attempt to delineate the processing order of the multiple intervening sequences.

Results

High Molecular Weight Ovalbumin and Ovomucoid RNAs Contain Poly(A)

As reported earlier, using the RNA blotting technique of Alwine et al. (1977) and probes obtained from cDNA clones of the ovalbumin and ovomucoid genes, we have demonstrated in oviduct nuclei the existence of multiple RNA species which contain ovalbumin or ovomucoid sequences and are larger than their respective mRNAs (Roop et al., 1978; Nordstrom et al., 1979). To determine whether these high molecular weight RNAs were polyadenylated we have isolated poly(A)$^+$ RNA from total oviduct nuclear RNA by oligo(dT)-cellulose chromatography. This poly(A)$^+$ RNA was electrophoresed in an agarose gel, transferred to diazobenzyloxymethyl (DBM) paper and hybridized to ^{32}P-labeled ovalbumin or ovomucoid cDNA clones (pOV230 and pOM100, respectively) (Figure

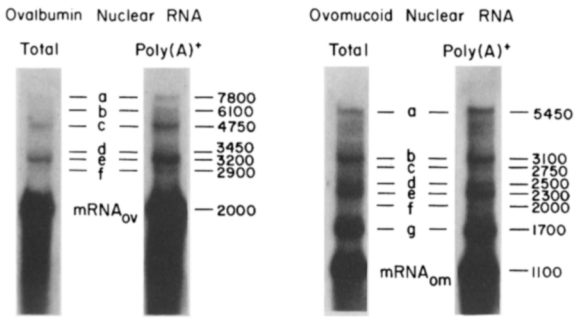

Figure 1. High Molecular Weight Forms of Ovalbumin and Ovomucoid RNA in Oviduct Nuclei Are Polyadenylated

Oviduct total nuclear RNA (20 μg) or poly(A) RNA (1 μg) was subjected to agarose gel electrophoresis in the presence of methylmercuryhydroxide and transferred to diazobenzyloxymethyl (DBM) paper as described in Experimental Procedures. The RNA covalently bound to the DBM paper was then hybridized to cloned ovalbumin cDNA (pOV230) or to cloned ovomucoid cDNA (pOM100) that was labeled with ^{32}P-dCTP and ^{32}P-TTP.

1). All the bands previously detected in total oviduct nuclear RNA were also observed in the poly(A)-containing RNA. Therefore, the vast majority of the high molecular weight ovalbumin or ovomucoid RNAs contain poly(A) sequences. Since no additional ovalbumin and ovomucoid high molecular weight bands were detected in the total RNA [as compared to poly(A)$^+$ RNA], the addition of poly(A) sequences must occur very rapidly after RNA chain completion.

Using single-stranded restriction enzyme fragments of λ DNA (RI and Hind III) as size standards, we determined the molecular size of these bands. As shown in Figure 1, the largest ovalbumin RNA band was estimated to have a size of 7.8 kb, which is four times larger than the mature mRNA$_{ov}$ and is ~200 nucleotides longer than the entire ovalbumin gene. (The length of the gene is defined in this case as the length between the 5′ and 3′ ends of the structural sequences.) Since this RNA band contains poly(A), the ~200 nucleotides of extra sequence could be accounted for by the poly(A) sequences at the 3′ end; therefore, the 7.8 kb RNA band may represent the primary transcript of the entire ovalbumin gene. Similarly, the largest ovomucoid RNA band has a size of 5.5–5.8 kb and is approximately six times larger than the mature ovomucoid mRNA. This band is also approximately 100–200 nucleotides longer than the entire ovomucoid gene. It should be noted that the bands designated as "a" through "g" are not the only bands observed; other minor bands do exist. For example, there are two minor bands between the "a" and "b" ovomucoid RNA bands and there is a faint band slightly larger than "g" band.

High Molecular Weight Ovalbumin RNAs Are the Precursors to Ovalbumin mRNA

Since the multiple high molecular weight ovalbumin and ovomucoid RNA bands contain both structural and intervening DNA sequences as previously reported (Roop et al., 1978; Nordstrom et al., 1979), we have suggested that these multiple RNA bands may represent the primary transcripts and various processing intermediates for the ovalbumin and ovomucoid mRNAs. To establish that these RNAs are indeed the precursors to mature RNA, kinetic labeling

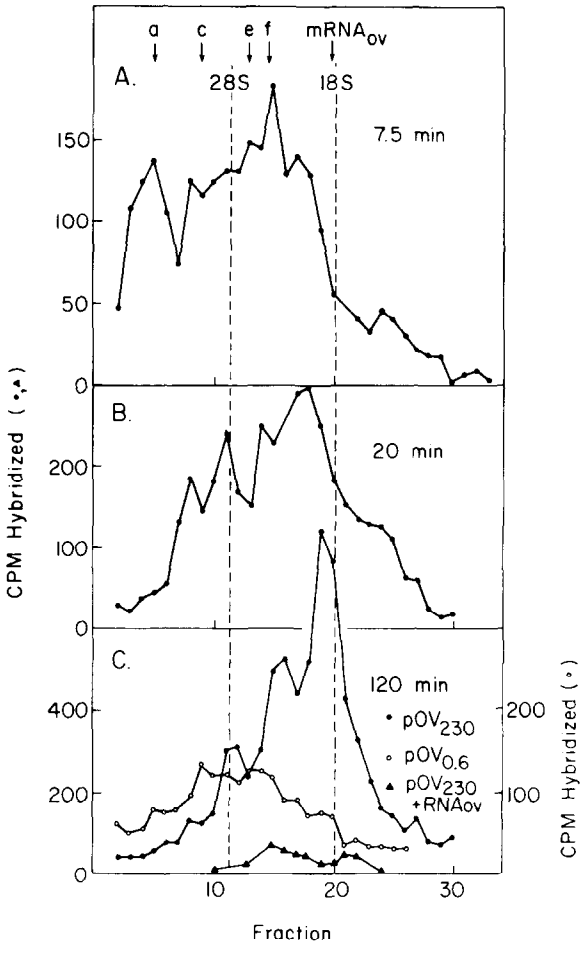

Figure 2. Kinetics of Labeling Nuclear Ovalbumin RNA

Labeling ^{3}H-oviduct nuclear RNA was carried out in oviduct tissue suspension according to a modification of the procedure of McNight (1978) as described in Experimental Procedures. ^{3}H-nuclear RNA isolated from the oviduct tissue suspension was then subjected to 1.4% agarose gel electrophoresis in the presence of 8 mM methylmercuryhydroxide as described in the legend to Figure 1. After electrophoresis, the gel was treated with 0.5 M ammonium acetate for 20 min at room temperature to remove the methylmercuryhydroxide. Hybridization and solubilization of the gel were carried out by a modification of the procedure of Schibler et al. (1978).

(A) 103 × 10^6 cpm ^{3}H-nuclear RNA from 6 g of oviduct tissue labeled for 7.5 min was applied to an agarose gel in three slots. The gel slices were solubilized in 500 λ of $NaClO_4$ buffer.

(B) 101 × 10^6 cpm of ^{3}H-nuclear RNA from 1.5 g of oviduct tissues labeled for 20 min was applied to an agarose gel in one slot. The gel slices were solubilized in 300 λ of $NaClO_4$ buffer.

(C) 70 × 10^6 cpm of ^{3}H-nuclear RNA from 0.75 g of oviduct tissue labeled for 120 min was applied to an agarose gel in one slot. The gel slices were solubilized in 300 λ of $NaClO_4$ buffer.

(–●–●–) ^{3}H-RNA hybridized to the pOV230 filter; (–○–○–) ^{3}H-RNA hybridized to the pOV0.6 DNA filter; (–▲–▲–) ^{3}H-RNA hybridized to the pOV230 filter in the presence of 10 μg of mRNA$_{ov}$.

and pulse-chase experiments in oviduct tissue-suspension systems have been carried out.

Freshly excised oviduct tissue cut into 0.5–2 mm slices was incubated in F-12 medium containing 1×10^{-8} M estradiol, 1 μg/ml of insulin and 0.5 mCi/ml of ^{3}H-cytidine and ^{3}H-uridine at 41°C. The ^{3}H-labeled nuclear RNA was extracted and subjected to agarose gel electrophoresis in the presence of the denaturing agent, methylmercury hydroxide. Gel slices were dissolved in $NaClO_4$ and the amount of ^{3}H-ovalbumin RNA sequences in each slice was then measured by hybridization to cloned ovalbumin DNA filters (pOV230). Analysis of ovalbumin RNA sequences present in nuclear RNA labeled for various times with ^{3}H-cytidine and ^{3}H-uridine is shown in Figure 2. When the oviduct suspension was labeled for 7.5 min at 41°C, the majority of the ^{3}H-RNA that hybridized to ovalbumin DNA had a molecular weight greater than 3000 nucleotides. Most importantly, there was no detectable labeling of mature ovalbumin mRNA sequences after this short incubation. The largest ovalbumin RNA molecules detected (Figure 2A, fraction 5–6) had a molecular weight of about 7.8 kb, which corresponds to band "a" of the steady state RNA (Figure 1). The RNA between 18S and 28S (Figure 2A, fraction 13–18) corresponds to the size of bands "d", "e" and "f" of steady state RNA, which are 3000–5000 nucleotides in length. When the tissue was incubated for a longer time the relative concentration of the highest molecular weight peak at 7.8 kb decreased and radioactivity corresponding to ovalbumin mRNA was detected (Figures 2B and 2C). After 120 min of incubation (Figure 2C) we estimate that approximately 70% of the hybridizable ^{3}H-RNA corresponds to mature ovalbumin mRNA. It should be emphasized that, while 6 g of oviduct tissues was needed to synthesize enough ^{3}H-RNA for an analysis of the 7.5 min incubation, only 1.5 and 0.75 g of oviduct tissues were used in the 20 and 120 min incubations, respectively. Therefore, while the relative concentration of ^{3}H-RNA hybridized at the 7.8 kb peak decreased as the incubation time was lengthened, the total incorporation of ^{3}H-RNA into ovalbumin sequences at this size actually increased with time.

To eliminate the possibility that the change in size during different labeling periods was due to a random cleavage of high molecular weight RNA instead of true excision of the intervening RNA sequences, the following experiments were carried out. First, ^{3}H-RNA, labeled for 120 min, was hybridized to DNA filters containing a cloned intervening sequence region of the ovalbumin gene. These filters contained no structural sequence DNA. If the low molecular weight RNA peak corresponding to the size of $mRNA_{ov}$ is derived from a random degradation of high molecular weight RNA, this peak should contain intervening DNA sequences as well as structural sequences and thus should hybridize with cloned intervening sequence DNA. In this case we used a cloned DNA sequence (pOV0.6) that consist of 0.6 kb of intervening sequence region "G" of the ovalbumin gene that was obtained by restriction enzyme digestion (Hae III and Eco RI) of pOV1.8. This DNA hybridized only with steady state high molecular weight ovalbumin RNA bands and not with mature $mRNA_{ov}$ (data not shown). When this DNA filter was used for hybridization only ^{3}H-RNA of high molecular weight hybridized to the 0.6 kb DNA filters, whereas no ^{3}H-RNA corresponding to the size of mature $mRNA_{ov}$ hybridized (Figure 2C). Second, the identity of the $mRNA_{ov}$ peak observed in these studies was further substantiated by hybridization competition experiments. The radioactive ovalbumin RNA sequences that hybridized the 18S region could be fully competed out by an excess of unlabeled $mRNA_{ov}$ (Figure 2C). Therefore, these control experiments indicate that the change in molecular weight ^{3}H-RNA during different labeling periods appears to be due to true processing events and

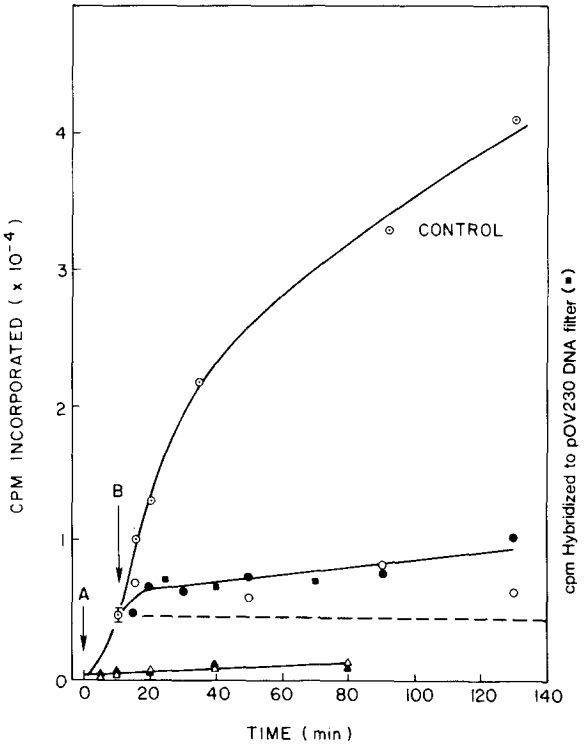

Figure 3. Inhibition of ^{3}H-RNA Synthesis by Excess Unlabeled Nucleosides and Actinomycin D

Incorporation of ^{3}H-RNA nucleosides into RNA was carried out in oviduct tissue suspension as described in the legend to Figure 2 for various times as indicated. At the end of incorporation the ^{3}H-RNA incorporated was measured by 20% trichloroacetic acid precipitation. (⊙) Control; 5 mM each or uridine and cytidine were added at (▲) 0 time or (●) 10 min after the start of reaction; 2 mM each of uridine and cytidine and 10 μg/ml of actinomycin D were added at (△) 0 time, or (○) 10 min after the start of reaction. Incorporation of ^{3}H into ovalbumin structural sequences was carried out by hydridizing the pulse-labeled and chased (with 5 mM C + U) ^{3}H-RNA to pOV230 DNA filters (■).

not to random degradation. This kinetic behavior of the nuclear RNA species containing ovalbumin RNA is consistent with a precursor-product relationship. The high molecular weight RNA (7.8 kb, band "a" of Figure 1) is first processed to RNA of medium size (bands "b" through "f" of Figure 1) and finally to mature $mRNA_{ov}$ (~2000 nucleotides in length).

One could still argue, however, for the possibility that high molecular weight components are synthesized and rapidly degraded without giving rise to $mRNA_{ov}$ molecules, while the mRNAs are synthesized independently at slower rates. To address this issue we carried out a pulse-chase experiment in which the cell was labeled with ^3H-uridine and ^3H-cytidine for 10 min and then chased with excess unlabeled nucleosides and actinomycin D. First we carried out experiments to demonstrate that in this chase experiment further ^3H-nucleoside incorporation was indeed inhibited during the chase period. As shown in Figure 3, after 10 min of incorporation the addition of 5 mM uridine and cytidine or 2 mM uridine and cytidine and 10 μg/ml actinomycin D inhibited further incorporation of ^3H-nucleosides. It should be noted also that excess uridine plus cytidine inhibited further incorporation of ^3H-nucleosides into ovalbumin structural sequences.

Next we carried out the pulse-chase experiment to find whether we could chase the pulse-labeled high molecular weight ovalbumin RNA into mature mRNA with an excess of unlabeled nucleosides and actinomycin D. As shown in Figure 4A, a 10-min incubation gave rise to high molecular weight RNA of 3.0–7.8 kb in length. After a 10 min incubation and 2 hr chase with excess unlabeled cytidine and uridine (Figure 4B), the majority of the hybridizable RNA existed in the region of mature $mRNA_{ov}$. Similarly, when actinomycin D was used to inhibit further RNA synthesis during the 2 hr chase period (Figure 4C), the majority of ^3H-RNA obtained had a size similar to $mRNA_{ov}$. Therefore, the high molecular weight RNA must be processed to give rise to 2000 nucleotide-long ovalbumin mRNA.

In this type of pulse-chase experiment it is important to demonstrate that ovalbumin RNA synthesized during the period of pulse labeling could be quantitatively chased into mature ovalbumin mRNA. According to our calculation approximately 4000 cpm of ovalbumin structural sequences have been synthesized in a 10 min pulse labeling period from 6 g of oviduct tissue (Figure 4A); after chasing with excess nucleosides in the presence or absence of actinomycin D ~8000 cpm of $mRNA_{ov}$ sequences are observed (Figures 4B and 4C). The 1 fold increase of ovalbumin mRNA sequences within the lengthy 2 hr chase period is consistent with the data given in Figure 3 which show that total RNA and $mRNA_{ov}$ synthesis also increased by 100% in this 2 hr period. This increase probably resulted from RNA synthesis which occurred before

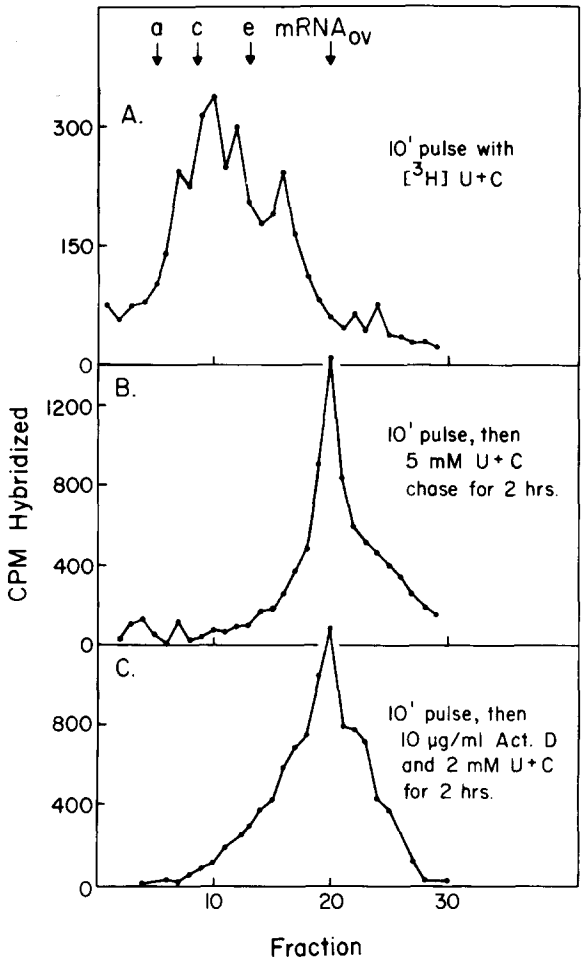

Figure 4. Pulse Labeling of Nuclear Ovalbumin RNA in Oviduct Tissue Suspension

Oviduct tissue suspension (6 g) was labeled with ^3H as described in the legend to Figure 2. After incubation for 10 min 5 mM each of uridine and cytidine (B) or 2 mM each of uridine and cytidine and 10 μg/ml of actinomycin D (C) were added to minimize further synthesis of ^3H-RNA. Incubations were then continued for an additional 2 hr. Conditions for isolation of ^3H-nuclear RNA, gel electrophoresis and hybridization were as described in the legend to Figure 2. The gel slices were dissolved in 200 λ of 2 M $NaClO_4$ buffer and hybridized to pOV230 DNA filters.

(A) 78 × 10^6 cpm of ^3H-nuclear RNA from tissue labeled for 10 min.

(B) 62.5 × 10^6 cpm of ^3H-nuclear RNA from tissue labeled for 10 min and then chased for 2 hr with 5 mM uridine and cytidine.

(C) 60 × 10^6 cpm of ^3H-nuclear RNA from tissue labeled for 10 min and chased for 2 hr with 2 mM uridine and cytidine and 10 μg/ml actinomycin D.

the conversion in oviduct nuclei of nucleosides to nucleotides required for an effective chase. Thus our results suggested that the vast majority of precursor RNA_{ov} synthesized in the 10 min pulse period must be converted into mature $mRNA_{ov}$.

High Molecular Weight Ovomucoid RNAs Are Precursors to Ovomucoid mRNA

Next we examined whether the high molecular weight ovomucoid RNAs were precursors to ovomucoid

mRNA. Newly synthesized nuclear RNA was labeled with ^3H for different periods of time in oviduct tissue suspension, size fractionated and analyzed for ovomucoid sequences by hybridization to DNA filters containing ovomucoid genomic DNA (pOM14). These clones contain all the ovomucoid gene sequences except for 120 bp of structural sequence at the 3′ end of the gene. The results of these experiments were similar to those observed for the ovalbumin RNAs (data not shown).

Therefore the kinetic labeling studies suggest that mature ovomucoid mRNA is derived from the higher molecular weight species of ovomucoid RNA. When pulse-chase experiments with actinomycin D were carried out for ovomucoid RNA sequences, once again the vast majority of the high molecular weight RNA could be chased into mature ovomucoid mRNA (Figure 5). Therefore, for both ovalbumin and ovomucoid genes, the mRNAs are synthesized as portions of high molecular weight precursors and these high molecular weight precursors containing both structural and intervening sequences are then processed to RNA that contains only structural sequences.

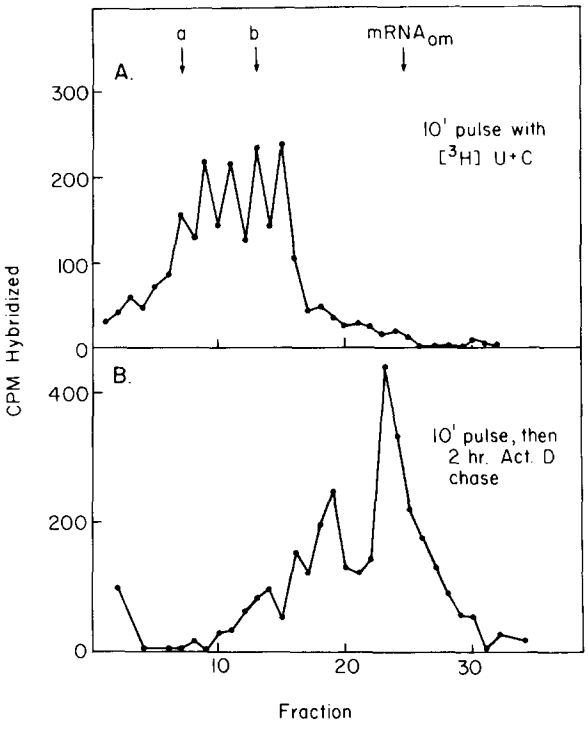

Figure 5. Pulse Labeling of Nuclear Ovomucoid RNA in Oviduct Tissue Suspension

Isolation and hybridization of ^3H-nuclear RNA to ovomucoid DNA filters were as described in the legend to Figure 3. 6 g of oviduct tissue were used for labeling in each experiment. The gel slices were dissolved in 300 λ of 2 M $NaClO_4$ buffer and hybridized to (A) pOM14 ovomucoid DNA filters or (B) pOM100 DNA filters.

(A) 105 × 10^6 cpm of ^3H-nuclear RNA from tissue labeled for 10 min.

(B) 60 × 10^6 cpm of ^3H-nuclear RNA from tissue labeled for 10 min, then chased for 2 hr with 2 mM each of cytidine and uridine and 10 μg/ml of actinomycin D.

Processing Pathway for Ovomucoid Precursor to mRNA

Our previous studies (Roop et al., 1978; Nordstrom et al., 1979) and the results described above indicate that the ovalbumin and ovomucoid genes are transcribed into high molecular weight RNA precursors and subsequently processed so that multiple processing intermediates are generated. We examined the pathway by which RNA precursors are processed into mature mRNA by analyzing the hybrids between these processing intermediates and the genomic DNA. A major question relative to RNA processing is whether the intervening sequences are spliced in an ordered or a random fashion. To answer this question we carried out an electron microscopic mapping study of heteroduplexes of pooled nuclear precursor RNA and genomic DNA. In this particular case we concentrated our efforts on ovomucoid precursors since they exist at a higher concentration within oviduct nuclei (Nordstrom et al., 1979). The ovomucoid RNAs, precursors as well as mRNA, were first enriched by hybridizing total nuclear RNA to DNA-DBM cellulose containing ovomucoid genomic DNA (pOM14). The RNA was then eluted from the cellulose. Analysis by agarose gel electrophoresis followed by transfer to diazobenzyloxymethyl paper and then hybridization to ^{32}P-ovomucoid cDNA (pOM100) revealed that the ovomucoid RNA enriched by pOM14 DNA cellulose was not significantly degraded (data not shown). Heteroduplexes were then formed by hybridizing the enriched ovomucoid RNA to heat-denatured OM14 DNA (pOM14 DNA that was digested with Eco RI to remove the pBR322 DNA sequences). OM14 DNA contains all of the ovomucoid natural gene sequences except for 120 nucleotide pairs of structural sequences at the extreme 3′ end of the gene (Lai et al., 1979). This cloned DNA also contains 8–9 kb of flanking DNA at the 5′ end of the structural sequences. It should be noted that the hybridization conditions we used will only allow RNA-DNA hybrid formation and not DNA-DNA reannealing. When the duplex between OM14 DNA and ovomucoid mRNA was analyzed by electron microscopy we observed structures containing seven loops (Figure 6A). In an earlier report (Catterall et al., 1979) we indicated that the ovomucoid structural gene was interrupted by at least six intervening sequences (Figure 6B). Detailed comparison of our present structure with that reported earlier revealed that the first loop in the 5′ region of our earlier report can be subdivided into two loops as shown in the diagram of Figures 6C and 6D. Therefore, there is a small structural sequence existing in the region between loops A and B which can be detected. The existence of a small structural sequence between loops A and B has been proven by direct sequence analysis (Lai et al., 1979). Using plasmid DNA as a size standard,

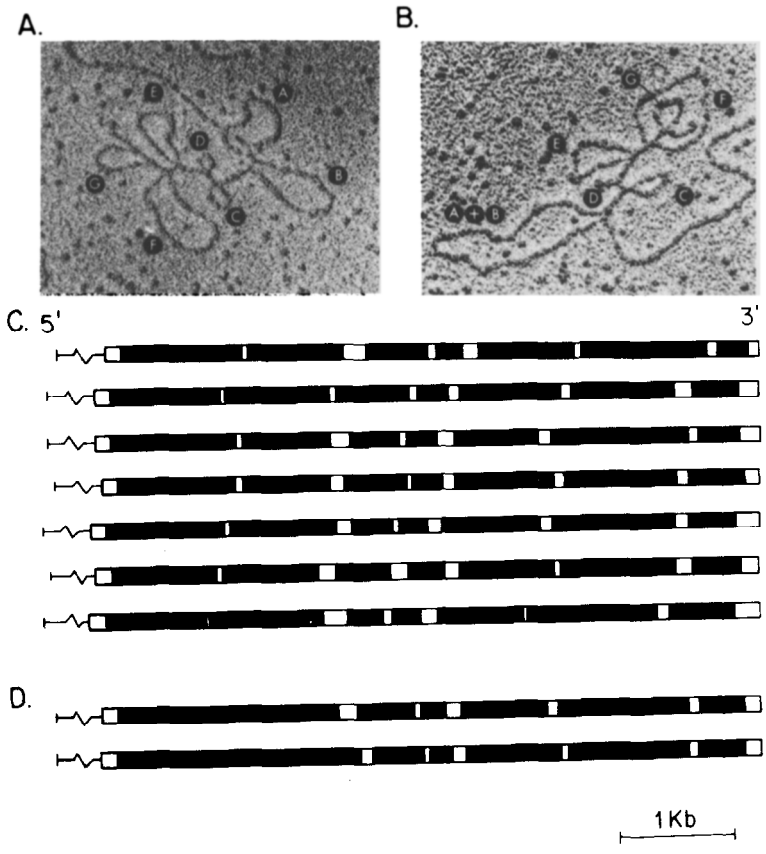

Figure 6. Electron Micrographs of the Hybrid Molecules between Ovomucoid mRNA and OM14 DNA

Ovomucoid RNA and pOM14 DNA (digested with Eco RI) were incubated as described in Experimental Procedures. The hybrids were viewed under Siemens Elmiskop 102A electron microscope.

(A) Hybrid between DNA and ovomucoid mRNA (7-loop structure).

(B) 6-loop structure.

(C) Diagrams of 7-loop heteroduplexes. Scale was determined by using the pBR322 plasmid sequence as the standard (4.36 kb). The standard deviations for all measurements were not more than 0.2 kb.

(D) Diagrams of 6-loop heteroduplexes.

we found the size of the ovomucoid gene to be 5.5 kb, which is higher than that estimated in our previous report (Catterall et al., 1979).

Further analysis of the hybrids between enriched ovomucoid nuclear RNA and OM14 DNA revealed a variety of DNA-RNA duplex structures representative of the population of nuclear ovomucoid RNA. A selection of these structures is shown in Figures 7 and 8. In addition to the 7-loop structures of the mature mRNA-DNA heteroduplex (Figure 6), we also observed DNA-RNA duplex structures containing varying combinations of loops (Figures 7 and 8). This observation is consistent with our earlier conclusion that these steady state RNA molecules are processing intermediates. It also supports the notion that these precursor RNA molecules contain covalently linked structural and intervening sequences.

Because the distance of each loop from the 3′ end of OM14 DNA (Eco RI cutting site) and the size of each loop are different, individual loops could be easily identified by measuring both their distance from the Eco RI site and their size in relation to the other loops. The existence of loops indicates that this particular intervening sequence in the RNA molecule is removed during the processing. Using this type of measurement we have identified all the loops in the DNA-RNA heteroduplexes (Figure 7). In all cases the measured length of the RNA-DNA hybrid shows the RNA moiety to be undegraded and intact. It should be emphasized that the molecules shown in this figure are not the only types of molecules observed. In addition to the molecules shown in Figure 7 we have observed molecules with different combinations of loops, two of which are shown in Figure 8. The existence of various combination of loops suggests that there may not be an absolute or obligatory pathway for removal of the intervening sequences.

Nevertheless, since certain combinations of loop structures were observed more frequently than others and some particular combinations of loop structures were not observed (for example, we have not observed a 1-loop structure having only loop B or C or D), the removal of intervening sequences appeared not to be a completely random process. To validate the existence of a preferred pathway(s) for removing the intervening sequences, we have examined more than 90 molecules of ovomucoid precursor RNA-DNA duplex structures and summarized the frequency of occurrence of certain loop structures. As shown in Table 1, out of 17 molecules which have had one intervening sequence removed, 5 of them had the E intervening sequence removed, 10 molecules had the F intervening sequence removed and only one molecule each had the G or A intervening sequence removed. None of the molecules observed revealed an initial removal of the B or C intervening sequences.

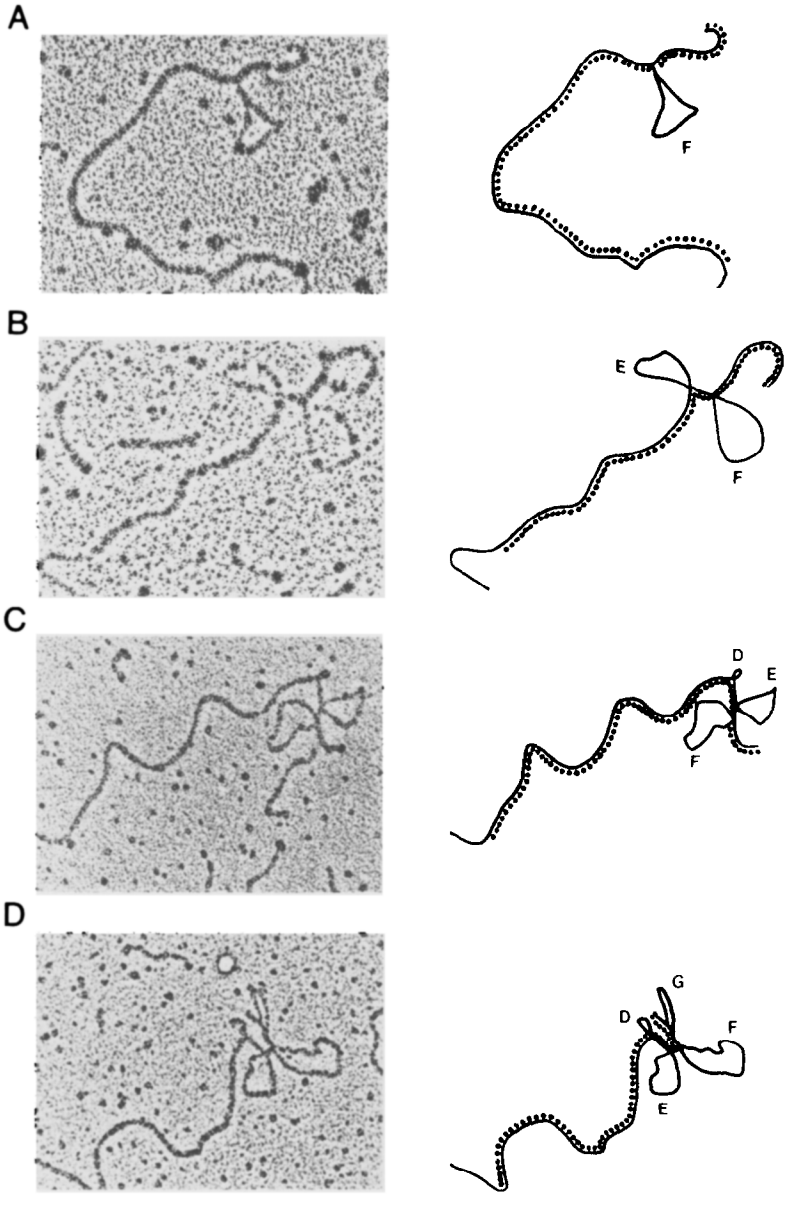

Figure 7. Electron Micrographs of the Hybrid Molecules between Ovomucoid RNA Precursors and OM14 DNA

(A) 1-loop hybrid with loop F. (B) 2-loop with loops E and F. (C) 3-loop hybrid with loops D, E and F. (D) 4-loop hybrid with loops D, E, F and G.

Similarly, out of 16 molecules of precursor RNAs which have had two intervening sequences removed, 10 molecules have E intervening sequences removed and 10 molecules have F removed. Only a few molecules have had other intervening sequences removed. When 3- or 4-loop structures are examined, the majority of these molecules have had E, F, D and G intervening sequences removed. In addition, however, a significant number of molecules also have had the A and B intervening sequences removed. In order to substantiate this preferred pathway we have carried out a control experiment to rule out the possible artifact of random degradation of RNA sequences. We have subjected mature ovomucoid mRNA to the same enrichment process. The hybrids formed between this treated mRNA and OM14 DNA contain most of the 7- and 6-loop structures, as shown in Figures 6A and 6B. From these results we propose that while removal of intervening sequences may not proceed in an obligatory fashion, there is a preferred pathway by which the intervening sequences are excised. This pathway is: (E and F) > (G and D) > (A, B and C).

Hybridization of Oviduct Nuclear RNA to Ovomucoid Gene Probes

To further test the hypothesis of a preferred processing pathway we analyzed the size of nuclear ovomucoid RNA molecules that hybridized to DNA probes prepared from different intervening sequence regions of the natural ovomucoid gene. Oviduct nuclear RNA was electrophoresed on agarose gels, transferred to diazobenzyloxymethyl paper and hybridized to the

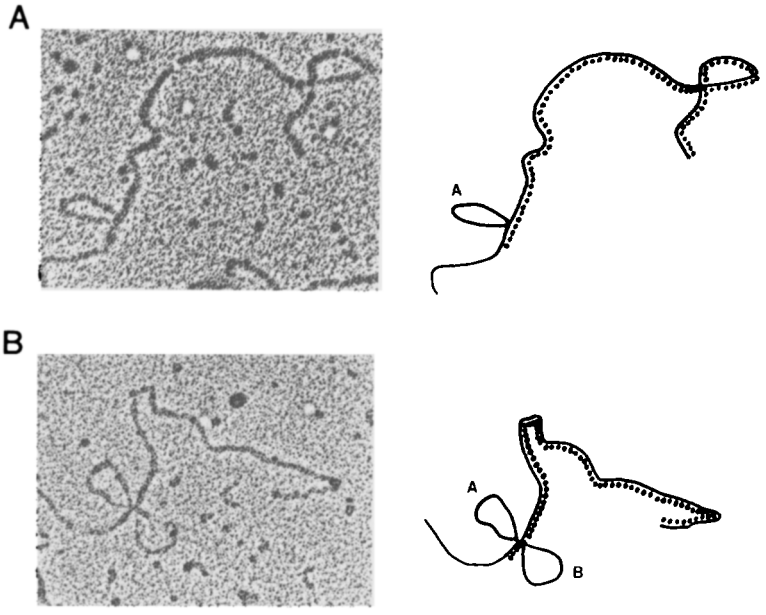

Figure 8. Electron Micrographs of the Hybrid Molecules between Ovomucoid RNA Precursors and OM14 DNA

(A) 1-loop hybrid with loop A. (B) 2-loop hybrid with loops A and B.

Table 1. Processing Intermediates of Ovomucoid RNA

Number of Loops per Duplex	Frequency of Molecules with Loop at Position							Total Number of Molecules Analyzed
	A	B	C	D	E	F	G	
1	1	0	0	0	5	10	1	17
2	3	3	0	2	10	10	4	16
3	1	1	1	6	20	19	12	20
4	2	5	7	19	18	18	11	20
5	5	9	8	10	12	9	7	12
6	5	9	7	8	9	9	7	9
Σ1–6	17	27	23	45	74	75	42	94

Only undegraded RNA-DNA hybrids in which the RNA moiety was shown to be intact were scored in this experiment.

^{32}P–DNA probes described in Figure 9. Probes specific for intervening DNA sequences E and F, which according to the electron microscopy data are the earliest to be excised, should hybridize mainly to the largest ovomucoid precursor(s). By contrast, probes specific for intervening DNA sequences A, B and C which appear to be the last to be excised should hybridize to most of the ovomucoid RNA intermediates (both large and small precursors). It should be noted some minor bands, the products of other minor processing pathways or deadend processing products, might also be hybridized to the E and F probes. The DNA probes corresponding to different intervening sequences were prepared from cloned DNA by cleavage with different restriction enzymes as illustrated in Figure 9. Since all these DNA fragments contained some structural sequence, hybridization reactions were performed in the presence of excess ovomucoid mRNA to minimize hybridization due to structural sequences. The lack of detection of mature ovomucoid mRNA by these probes indicates that the conditions for competition were sufficiently complete (Figure 9). As shown in Figure 9, pOM1.1 and pOM0.8 hybridized to most of the ovomucoid precursor bands detected by the ovomucoid cDNA probe pOM100. pOM1.1 contains intervening sequence A and approximately 200 nucleotides of intervening sequence B; pOM0.8 contains the remaining portion of the intervening sequence B and 100–200 nucleotides of intervening sequence C. In contrast, both OM 0.52, which contains only intervening sequence E, and OM 1.08, which contains only intervening sequence F, hybridized mainly to the largest ovomucoid RNA (band "a"). Therefore, E and F intervening sequences must be removed earlier during RNA processing. In addition to the "a" band, after long exposure to these two probes (especially F), we detected some minor bands. These minor bands may derive from a minor processing pathway or dead-end processing products. These results indicated once again that a preferred pathway

existed in the processing of ovomucoid gene transcripts. This preferred pathway is E, F > D, G > A, B, and C.

Removal Kinetics of Intervening Sequences from Nuclear Poly(A)-Containing RNA

To confirm further that a preferential processing pathway does exist for ovomucoid precursor RNA, we carried out pulse-chase experiments on oviduct nuclear RNA to study the rate at which individual intervening sequences are removed from high molecular weight ovomucoid RNAs. Poly(A) RNA was used for this study so that free intervening sequences which have been spliced out from precursors will not interfere with the interpretation of our results. Poly(A) RNAs were isolated from the nuclei of oviduct tissue slices which were either pulse-labeled and then chased with 5 mM unlabeled cytidine and uridine for varying periods of time. The concentration of individual intervening sequences in the different samples was then measured by hybridizing the sequences in the ^{3}H-RNA to plasmid DNA filters containing various intervening DNA sequences. The results of these experiments are expressed as percentages of sequence relative to that of the pulse-labeled sample versus time of chase (Figure 10). During the chase the intervening sequences E and F were removed faster from nuclear poly(A) RNA than that of the intervening sequences A, B and C. The half-life for this process was approximately 10–30 min. As expected, the concentration of the structural sequences measured by hybridization to cDNA filters (pOM100) did not change significantly.

Taken together, these results strongly support our conclusion based on the electron microscope mapping and Northern hybridization: removal of the multiple ovomucoid intervening sequence does not proceed in a random manner, but rather through a preferred pathway. The preferred pathway appears to involve first the removal of intervening sequences E and F, followed by the removal of intervening sequences D and G and finally by the removal of intervening sequences A, B and C.

Discussion

The data contained in the present report demonstrate that the high molecular weight ovalbumin and ovomucoid RNAs found in oviduct nuclei are precursors to their respective mRNAs, as we have suspected previously (Roop et al., 1978; Nordstrom et al., 1979). The largest detectable ovalbumin precursor RNA (7.8 kb) is slightly larger than the ovalbumin gene, which spans approximately 7.6 kb (from the cap site at the 5′ end to the poly(A) addition site at the 3′ end). We recently, have assayed the steady state concentration in oviduct nuclear RNA sequences complementary to the DNA within the ovalbumin gene as well as to the DNA flanking the gene at its 5′ and 3′ ends (Tsai et al., 1980). We found that there was less than one copy, if any, of transcripts corresponding to the flanking DNA per oviduct nucleus. Similarly, using pulse-labeled RNA from oviduct nuclei or oviduct tissue suspensions in vitro, we also failed to observe any transcription from flanking DNA sequences. These

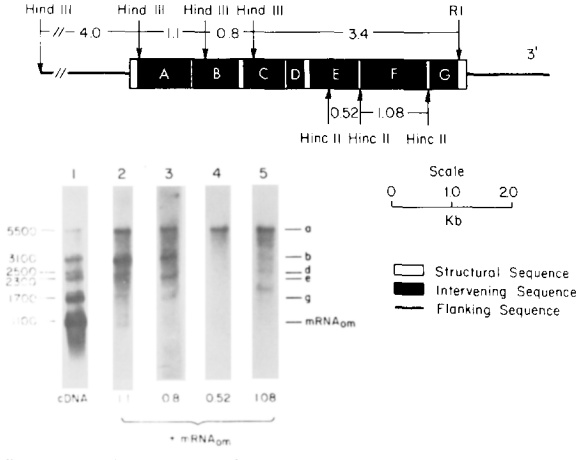

Figure 9. Hybridization of Oviduct Nuclear RNA to Ovomucoid DNA Probes

The DNA fragments (pOM100, pOM1.1, pOM0.8, OM0.52 and OM1.08) were labeled with ^{32}P-dCTP and ^{32}P-TTP to a specific activity of 5–10 × 10^{8} cpm/μg as described in the legend to Figure 1. Electrophoresis and transfer of oviduct nuclear RNA to diazobenzyloxymethyl (DBM) paper were carried out as described in the legend to Figure 1, except that the denatured ^{32}P-labeled DNA probes were incubated with 20 μg of ovomucoid mRNA at 42°C for 20–30 min prior to the hybridization to the nuclear RNA covalently linked to DBM paper.

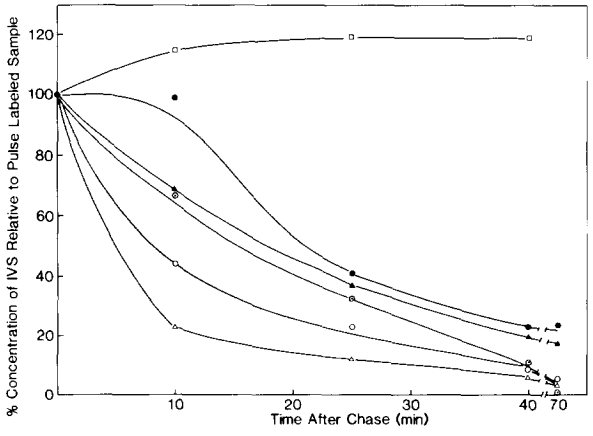

Figure 10. Turnover of Ovomucoid Intervening Sequences from Nuclear Poly(A)-RNA

^{3}H-poly(A)-RNA was isolated from nuclei of oviduct tissue slices which were pulse-labeled or pulse-labeled and then chased with 5 mM each of uridine and cytidine for 10, 25, 40 and 70 min. The ^{3}H-RNAs were then hybridized to DNA filters containing cloned individual intervening sequences and 10 μg of mRNAom. The filters contained (□) pOM100 (cDNA); (●) pOM0.6 (intervening sequence A); (▲) pOM0.8 (intervening sequences B + C); (○) pOM0.52 (intervening sequence E); △ pOM1.08 (intervening sequence F); and (⊙) pOM0.65 (intervening sequence G).

observations are consistent with our failure to detect the presence of ovalbumin RNA sequences larger than the 7.8 kb band in either steady state or pulse-labeled RNA. Therefore the largest RNA band detected (7.8 kb) appears to represent the primary transcript of the ovalbumin gene. Similar to the ovalbumin gene, the largest detectable ovomucoid RNA precursor (5.5–5.8 kb) contains sequences complementary to both structural and intervening DNA sequences and is only slightly larger than the ovomucoid gene. This RNA band may also represent the primary transcript of the ovomucoid gene.

During pulse labeling of oviduct tissue suspensions we detected some ovalbumin RNA molecules the size of bands "d", "e" and "f" (in addition to the 7.8 kb RNA band) even at the shortest times. This may be due to a rapid processing of the first few intervening sequences which occurs either before or shortly after the completion of the entire RNA chain. Incubation for times shorter than 7.5 min is technically difficult to perform since large amounts of ^{3}H-labeled RNA are required for hybridization after gel electrophoresis. However, the detection of the 3.0–3.5 kb RNAs in the RNA labeled for short periods should not affect our conclusion since both the 7.8 kb RNA and 3.0–3.5 kb RNAs could be chased into mature $mRNA_{ov}$.

Both electron microscopic mapping of the ovomucoid processing intermediates and hybridization of oviduct nuclear RNA covalently bound to diazobenzyloxymethyl paper by ovomucoid DNA probes specific for individual intervening sequences indicated that removing intervening sequences from ovomucoid precursor RNAs is carried out in a preferred pathway. This conclusion was further supported by the different processing rate by which individual intervening sequences were removed from precursor RNAs during pulse-chase experiments. The detection of a minor band by intervening sequences E and F and the observation of alternate loop combinations in the RNA-DNA hybrids examined by electron microscopy may suggest the existence of a minor processing pathway. Alternatively, this small number of RNA molecules may represent dead-end processing products.

We have attempted to correlate the banding pattern of ovomucoid precursor observed using the Northern transfer technique with our predicted pathway for removal of individual intervening sequences. It should be emphasized that due to the lack of proper RNA standards at the high molecular weight size range, the sizes of the Northern bands relative to DNA standards are only a rough estimation. The size estimates are generally smaller than those calculated using rRNA standards. In addition, due to the lack of complete sequence data for the ovomucoid natural gene, the exact lengths of intervening sequences are not known. With these two points in mind, we estimated that removal of intervening sequences E and F should generate an RNA band ~2 kb shorter than the primary transcripts. Next, removing intervening sequences D and G should further shorten the RNA by ~0.75 kb. As shown in Figures 1 and 9 there are two major intermediates, one at or slightly larger than 3.1 kb (band b) and the other one at 2.2–2.5 kb (band d or e). These two bands correspond roughly to the precursor intermediates lacking intervening sequences E + F and D + E + F + G, respectively. Another major precursor RNA band at the 1.6–1.8 kb region (band g) appears to represent the precursor containing only intervening sequence C, since the size difference between band "g" and $mRNA_{om}$ is close to that of intervening sequence D. This latter correlation has been confirmed by hybridization studies which indeed show that this final intermediate contains intervening sequence C.

In conclusion, we have demonstrated the existence of precursor RNAs to ovalbumin and ovomucoid mRNAs in oviduct nuclei. These RNAs are labeled preferentially at short-time incubations in oviduct suspension culture and are then converted into mature mRNAs. While there are numerous potential combinations in removing the intervening sequences during processing the precursors to mRNA, certain pathways appear to be preferred. If one assumes that a single or limited number of enzyme systems is involved in precursor maturation, the data suggest that the structural configurations of RNA precursors can influence processing kinetics.

Experimental Procedures

Materials

^{3}H-uridine (46 Ci/mmole), ^{3}H-cytidine (29 Ci/mmole), ^{32}P-dCTP (345 Ci/mmole) and ^{32}P-dTTP (345 Ci/mmole) were purchased from Amersham. Actinomycin D was obtained from CalBiochem. F-12 medium was obtained from Gibco.

Isolation of DNA Probes Corresponding to Different Regions of Natural Gene

pOM1.1, pOM100, pOM0.8, pOM3.4, pOM14, pOV230 and pOV0.6 chimeric plasmids were constructed in our laboratory as described previously (McReynolds, Catterall and O'Malley, 1977; Stein et al., 1978; Catterall et al., 1979; Lai et al., 1979). The OM0.52 DNA fragment was obtained by Hinc II endonucleolytic cleavage of pOM3.4 DNA according to the procedure described previously (Roop et al., 1978; Roop, Tsai and O'Malley, 1980). The OM1.08 DNA fragment was obtained by digesting pOM3.4 DNA with Hinc II and Eco RI enzyme. After restriction enzyme digestion these two DNA fragments were purified twice by electrophoresis through 1% agarose gels. These DNAs were then labeled with ^{32}P-dCTP and ^{32}P-TTP to a specific activity of 5–10 × 10^{8} cpm/μg as described (Roop et al., 1978).

Electrophoresis of Nuclear RNA on Methylmercuryhydroxide Agarose Gel

Oviduct nuclear RNA, isolated from chicks stimulated chronically with diethylstilbestrol as described previously (Roop et al., 1978, Tsai et al., 1978) was subjected to oligo(dT)-cellulose chromatography according to the procedure of Aviv and Leder (1972). The poly(A)$^{+}$ fraction was rechromatographed according to the same procedure. Total nuclear RNA (20 μg) or nuclear poly(A)$^{+}$ RNA (1 μg) was then subjected to agarose gel electrophoresis in the presence of methylmercuryhydroxide and transferred to diazobenzyloxymethyl (DBM)

paper as previously described (Roop et al., 1978). The RNA covalently bound to the DBM paper was then hybridized to cloned ovalbumin cDNA (pOV230), to cloned ovomucoid cDNA (pOM100) or different DNA fragment of ovomucoid gene that were labeled with ^{32}P-dCTP and ^{32}P-TTP as described previously (Roop et al., 1978). Size of the individual band was calculated by comparison to the electrophoretic mobility of single-stranded DNA standards. DNA standards were obtained by digestion of λDNA with Eco RI (21.75, 7.52, 5.92, 5.52, 4.79 and 3.38 kb) or Hind III (23.2, 9.73, 6.46, 4.49, 2.30, 2.0 and 0.68 kb). It should be noted that when ribosomal RNAs (chicken 27S, 4.53 kb; chicken 18S, 2.08 kb; E. coli 16S, 1.5 kb; E. coli 30S, 3.13 kb) were used as standards, the sizes of the RNA bands greater than 18S were estimated to be slightly larger than that stated in the text (100–300 nucleotides larger).

Preparation of DNA Filters

DNA filters were prepared with cloned DNA of pOV230 and pOM100, chimeric plasmids containing ovalbumin and ovomucoid cDNA respectively, as previously described (McReynolds et al., 1977; Stein et al., 1978). Filters were also prepared with pOM14 DNA, ovomucoid genomic DNA clone, pOV0.6, a chimeric plasmid containing 0.6 kb of the G intervening sequence region of the ovalbumin gene (Catterall et al., 1979), pOM0.6, a chimeric plasmid containing 0.6 kb of the A intervening sequence of the ovomucoid gene (Lai et al., 1979), pOM1.08, a chimeric plasmid containing 1.08 kb of intervening sequence F, pOM0.52, a chimeric plasmid containing 0.52 kb of intervening sequences E and pOM 0.65, a chimeric containing intervening sequence G.

Detection of Ovalbumin or Ovomucoid Sequences in ^{3}H-RNA Labeled in Oviduct Tissue Suspension

Labeling of ^{3}H-oviduct nuclear RNA was carried out in oviduct tissue suspension according to a modified procedure of McKnight (1978). Oviducts from DES-stimulated chicks were finely minced at room temperature in Coon's-Bordelon F-12 nutrient mixture containing penicillin (100 units/ml), streptomycin (100 μg/ml), $NaHCO_3$ (2.5 mg/ml), insulin (1 μg/ml) and estradiol (10^{-8} M). Reaction mixtures (7.5 ml per flask) were incubated at 41°C for the times indicated in plastic flasks containing 0.2 g/ml oviduct tissue, 0.67 mCi/ml ^{3}H-cytidine (29 Ci/mmole) and 0.33 mCi/ml ^{3}H-uridine (46 Ci/mmole). The flasks were gassed with a mixture of 5% CO_2 and 95% O_2. At the end of the incubation period the reaction was stopped by the addition of cold 5% citric acid, and RNA was isolated from purified nuclei as previously described (Tsai et al., 1978). In the pulse-chase experiments ^{3}H-RNAs were pulse-labeled for 10 min, then chased with either 5 mM each of U and C or 2 mM each of U and C and 10 μg/ml actinomycin D. The tissues were further incubated for another 2 hr. Isolated ^{3}H-nuclear RNA (60–105 × 10^{6} cpm) and molecular weight standards were subjected to 1.4% agarose gel electrophoresis in the presence of 8 mM methylmercuryhydroxide as described earlier. After electrophoresis the gel was treated with 0.5 M ammonium acetate for 20 min at room temperature to remove the methylmercuryhydroxide. Hybridization and solubilization of the gel were carried out by a modified procedure of Schibler et al. (1978). Briefly, the gel was sliced into 2 mm slices and dissolved in 200–500 μl of 2 M $NaClO_4$, 50 mM Tris-HCl (pH 7.5), 10 mM mercaptoethanol, 250 μg/ml of yeast RNA, 0.1% SDS, 0.02% BSA, 0.02% polyvinylpyrrolidone and 0.02% Ficol at 71°C for 20–30 min. Filters containing 2–4 μg of pOV230, pOV230, pOV0.6, pOM14 or pOM100 DNA were added and the hybridized reaction was carried out between 65°C and 68°C for 16–20 hr. The filters were then washed according to the procedure described by Schibler et al. (1978).

Electron Micrography of the Hybrid Molecules between Ovomucoid mRNA and OM14 DNA

Ovomucoid RNAs were enriched by hybridizing oviduct nuclear RNA to pOM14 DNA-DBM cellulose according to the procedure of Child, Levy and Kedes, (1979) except that hybridization was carried out at 42°C and ovomucoid RNA was eluted with H_2O at 80°C. Hybrids between ovomucoid-enriched RNA and OM14 DNA (Eco RI-digest pOM14 DNA) were prepared as follows. 20 μg/ml of DNA and 6 μg/ml of RNA in 20–40 μl of 70% formamide, 0.1 M Tris-HCl (pH 8.8), 10 mM EDTA, and 0.3 M NaCl were denatured by heating at 80°–90°C for 2 min. The samples was then hybridized at 55°C for 4–6 hr. Aliquots were prepared for spreading by diluting with 2 vol of 70% formamide, 10 mM EDTA and 20 μg/ml cytochrome C. The hybrids were picked up on collodion coated grids and shadowed with platinum-palladium and carbon. The hybrids were viewed under Siemens Elmiskop 102A electron microscope.

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