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Evidence That Deoxyribonucleic Acid Sequences Flanking the Ovalbumin Gene Are Not Transcribed†

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ABSTRACT: The transcription of DNA sequences flanking the 5′ end and 3′ end of the ovalbumin gene was examined. First, various restriction endonuclease fragments corresponding to the 5′ and 3′ regions of the gene were isolated and used as hybridization probes to assay for the presence of transcripts corresponding to these different regions in the chick oviduct nuclear RNA. Very little, if any, of the transcripts corresponding to sequences flanking the 5′ and 3′ structural sequences of the ovalbumin gene was detected in the steady-state nuclear RNA. Second, RNA was pulse labeled either in isolated nuclei or in an oviduct tissue suspension system and hybridized to DNA filters containing purified fragments of various 5′- and 3′-flanking regions. Our results again demonstrated that RNA was not synthesized from the 5′- and 3′-flanking regions surrounding the gene. Taken together, these results are consistent with the postulate that flanking DNA sequences are not transcribed and that the largest RNA species detected in the nuclear RNA are the initial transcripts.

The natural ovalbumin gene, ~7.6 kb in length, is composed of eight segments of structural DNA sequences and seven intervening sequences (Dugaiczyk et al., 1978a,b, 1979; Woo et al., 1978; Garapin et al., 1978; Lai et al., 1978; Mandel et al., 1978; Gannon et al., 1979). In order to understand how the ovalbumin gene is transcribed, we have demonstrated in our initial studies that structural and intervening sequences are transcribed at similar rates in vitro (Roop et al., 1978). However, the concentration of RNA transcripts for structural sequences is 10-fold higher than that of transcripts for intervening sequences in steady-state nuclear RNA. Also, transcripts for intervening sequences are not detected in polysomal RNA (Tsai et al., 1979). These results suggested that the entire ovalbumin gene may be transcribed as a large precursor and the intervening sequences are subsequently removed and metabolized to give rise to stable mature $mRNA_{ov}$.

Further studies demonstrated that transcripts of various lengths which are larger than mature $mRNA_{ov}$ and contain sequences homologous to both structural and intervening sequences of the ovalbumin gene have been found in oviduct nuclear RNA (Roop et al., 1978). The largest of these molecules detected by Northern blotting techniques was 7.8 kb in length, which is very similar to the size of the ovalbumin natural gene. These high molecular weight RNAs can be pulse labeled in an oviduct tissue suspension system, and the radioactivity can be chased into mature mRNA in the presence of an excess of unlabeled nucleoside or actinomycin D (M.-J. Tsai, A. C. Ting, J. L. Nordstrom, and B. W. O'Malley, unpublished experiments). These results support our view that the high molecular weight ovalbumin RNA detected might indeed be the precursor to mature $mRNA_{ov}$.

The absence of ovalbumin nuclear RNA larger than 7.8 kb in length either in steady-state or in pulse-labeled RNA suggested that the initiation and termination of the ovalbumin gene transcripts may lie close to the 5′ end (cap site) and 3′ end [poly(A) addition site] of the mRNA. Alternatively, the ovalbumin gene could be transcribed as a precursor larger than 7.8 kb and then rapidly processed to form the 7.8-kb RNA. To develop evidence which would distinguish between these possibilities, we designed experiments which would define the existence or absence of RNA transcripts corresponding to DNA sequences flanking both 5′ and 3′ ends of the ovalbumin gene in either steady-state or pulse-labeled oviduct nuclear RNA.

Materials and Methods

Materials

Oviducts were obtained from white Leghorn chicks. The chicks were implanted subcutaneously each week with a 20-mg diethylstilbestrol (DES) pellet (Sigma Chemical Co.) which provided continuous release of DES for 8 to 9 days. Restriction endonucleases were purchased from Bethesda Research Lab-

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oratories. S_1 nuclease was obtained from Miles Laboratories. DNA polymerase I was a product of Boehringer. [^{3}H]dCTP and [^{3}H]dTTP were purchased from New England Nuclear Corp. Other radioactive compounds were purchased from Amersham Corp. DNase (DPFF) was obtained from Worthington. All chemicals were reagent grade and were purchased from Fisher Scientific Co.

Methods

Restriction Endonuclease Digestion. Restriction endonuclease digestions were carried out according to the recommended assay conditions of the supplier and contained 1 unit of enzyme per μg of DNA.

Electrophoresis and Extraction of DNA from Agarose Gels. Two hundred and fifty micrograms of DNA was applied to a 0.6-cm thick 1% agarose slab gel, and electrophoresis was carried out in a Tris–acetate buffer as previously described (McReynolds et al., 1977). After electrophoresis, the location of the DNA fragments was determined by staining with ethidium bromide. Agarose containing the DNA fragment was passed through a 21-gauge needle, suspended in 2 volumes of extraction buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 1 mM EDTA), and incubated at 37 °C overnight. Agarose was pelleted by centrifuging the gel suspension in an SW40 rotor for 2 h at 35000 rpm. The supernatant was extracted in an ice–water bath with an equal volume of redistilled phenol saturated with extraction buffer. After centrifugation, the aqueous layer was precipitated with ethanol. The precipitate was resuspended in 10 mM Tris, pH 7.6, and 1 mM EDTA, made 200 mM in NaCl, and extracted twice with phenol as described above. The aqueous layer was then extracted with chloroform–isoamyl alcohol (24:1) and precipitated with ethanol.

Labeling of DNA by Nick Translation. DNA was labeled by nick translation using a modification of the procedure previously described (Roop et al., 1978). The reaction was performed in a final volume of 100 μL containing the following: 50 mM Tris (pH 7.8), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 5 μg of BSA, and 1 μg of DNA. For [^{3}H]DNA the reaction mixture also contained 0.12 mM dATP and dGTP and 0.02 mM [^{3}H]dCTP and [^{3}H]dTTP (25.6 and 53 Ci/mmol, respectively, evaporated to dryness). For [^{32}P]DNA, the reaction mixture also contained 0.06 mM dATP and dGTP and 0.009 mM [^{32}P]dCTP and [^{32}P]dTTP (7–1000 Ci/mmol each). These components were assembled on ice and then 0.05 ng of DNase was added. The mixture was incubated at room temperature for 1 min and immediately cooled in an ice–water bath. Twenty microliters of *Escherichia coli* DNA polymerase I (80 units) was added, and the mixture was incubated at 14 °C. The reaction was stopped after 3 to 4 h of incubation by the addition of 100 μL of 0.2 M EDTA and 100 μg of *E. coli* DNA and heating at 68 °C for 10 min. Unincorporated dNTPs were separated from the labeled DNA by gel filtration on a Sephadex G-50 column using 10 mM Tris (pH 7.5), 1 mM EDTA, and 10 mM NaCl. Radioactive fractions eluting in the void volume were pooled, made 0.2 M in NaCl, and precipitated by the addition of 2 volumes of ethanol. The specific activity of [^{3}H]DNA was $(4–6) \times 10^7$ cpm/μg, and that of [^{32}P]DNA was $(5–10) \times 10^8$ cpm/μg.

Isolation of Nuclear RNA and RNA–DNA Hybridization. Nuclear RNA was isolated from nuclei, and RNA excess hybridization was performed as previously described (Roop et al., 1978).

DNA Transfer and Hybridization of [^{3}H]RNA. DNA was electrophoresed on a 1% agarose slab gel using an apparatus manufactured by Blair Craft (Cold Spring Harbor, NY). The DNA was then transferred to nitrocellulose fibers by the method of Southern (1975). After the transfer the filter was baked at 68 °C for 4 h and then for 16 h in 6 × SSC containing 0.04% ficoll, poly(vinyl)pyrrolidone, and bovine serum albumin. The filter was hybridized with ^{32}P-labeled RNA in the same solution containing 0.5% $NaDodSO_4$ and 1 mM EDTA for 36 h at 68 °C. After hybridization the filter was washed 4 times in 1 × SSC + 0.5% $NaDodSO_4$ at 68 °C for a total of 6 h. The filter was then exposed to X-ray film in the presence of a Du Pont Cronex intensifying screen at −20 °C.

In Vitro RNA Synthesis in Isolated Nuclei. Nuclei were isolated from oviducts obtained from chicks chronically stimulated with diethylstilbestrol as previously described (Towle et al., 1977) and used for in vitro RNA synthesis. [^{3}H]RNA was synthesized under the following conditions: 80 mM Tris-HCl, pH 7.9; 2.5 mM $MnCl_2$; 3 mM $MgCl_2$; 100 mM $(NH_4)_2SO_4$; 0.6 mM each of ATP, GTP, and UTP; 25 μM CTP (20 Ci/mmol); 30 μM EDTA; 6 mM dithiothreitol; 400 μg/mL DNA; 15% glycerol. [^{32}P]RNA was synthesized under the same conditions except that 1 mM each of GTP, UTP, and CTP and 40 μM ATP (20 Ci/mmol) were used. Reactions were incubated at 37 °C for specified time periods and then treated with affinity column purified DNase I (Maxwell et al., 1977) (20 μg/mL) for 15 min at 37 °C. The digestion was terminated by the addition of EDTA, $NaDodSO_4$, and proteinase K to final concentrations of 5 mM, 0.5%, and 20 μg/mL, respectively. The [^{3}H]RNA was then extracted as described for nuclear RNA, except that only one additional treatment with DNase was employed.

Synthesis of [^{3}H]RNA in Tissue Suspensions. Tissue suspensions were incubated by the procedure of McKnight (1978) as modified by Tsai et al. (unpublished experiments). Oviducts from DES-stimulated chicks were finely minced at room temperature in Coon's-Bordelon F-12 Nutrient Mixture (GIBCO) containing penicillin (100 units/mL), streptomycin (100 μg/mL), $NaHCO_3$ (2.5 mg/mL), insulin (1 μg/mL), and estradiol (10^{-8} M). Incubations were carried out for 5 or 10 min at 41 °C in plastic flasks containing tissue at 0.2 g/mL, [^{3}H]cytidine (29 Ci/mmol) at 0.67 mCi/mL, and [^{3}H]uridine (46 Ci/mmol) at 0.33 mCi/mL. The flasks were gassed with 5% CO_2 and 95% O_2. The synthesis was stopped by the addition of cold 5% citric acid, and RNA was isolated from purified nuclei as previously described (Roop et al., 1978).

In Vitro Synthesis of $RNA_{3.2}$ and $RNA_{2.7}$. RNAs were transcribed from their corresponding templates [$RNA_{3.2}$ (OV3.2) and $RNA_{2.7}$ (OV2.7)] by *E. coli* RNA polymerase (isolated as previously described; Towle et al., 1977) by using the following conditions: 50 mM Tris-HCl, pH 7.9; 5 mM $MgCl_2$; 100 mM $(NH_4)_2SO_4$; 2 mM 2-mercaptoethanol; 0.8 mM sodium phosphate; 5 μg/mL DNA template; 1 mM each of ATP, GTP, CTP, and UTP; 300 μg/mL *E. coli* RNA polymerase. Synthesis was for 2 h at 37 °C. The termination of the reaction and the extraction of RNA were as described above.

Hybridization of [^{3}H]RNA to DNA Filters. [^{3}H]RNA [$(1–6) \times 10^6$ cpm] and competitor RNA, where indicated (10 μg of $RNA_{3.2}$ or 10 μg of $RNA_{2.7}$), were dissolved in water and heated at 100 °C for 5 min to denature any double-stranded sequences. The RNA was then quick cooled in an ice–water bath. The RNA solution was made 0.6 M in NaCl, 10 mM in Hepes, pH 7.0, and 2 mM in EDTA (final volume 50 μL). The reaction was started by the addition of DNA filters (1.2 μg/filter); incubations were carried out at 68 °C for 18 h. Hybridization reactions were covered with paraffin

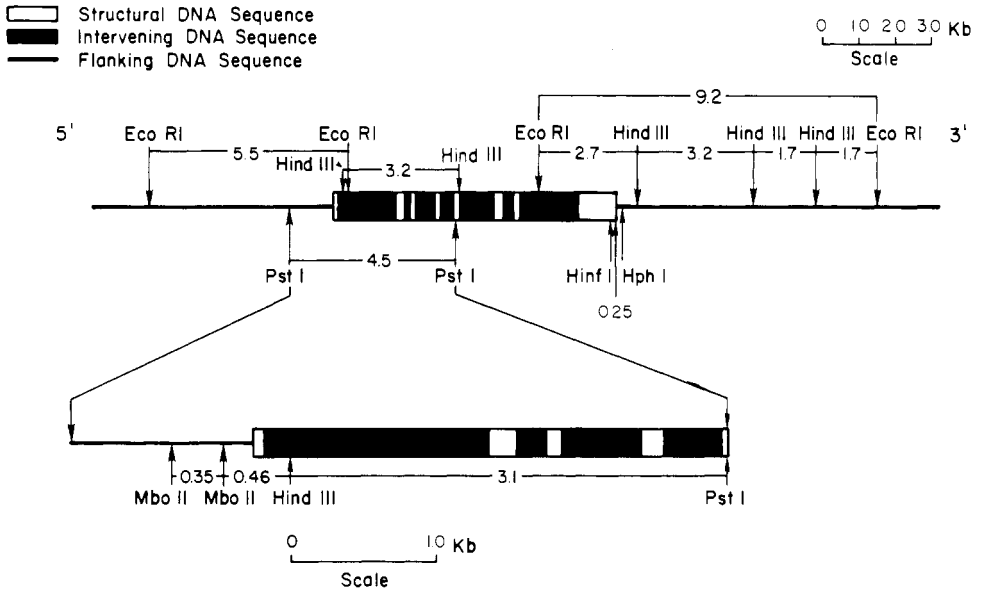

FIGURE 1: Map of the natural ovalbumin gene displaying the restriction endonuclease cleavage sites used in the construction of probes.

oil to prevent evaporation during the incubation. At the end of the incubation, filters were removed and rinsed in 2 × SSC and 0.1% $NaDodSO_4$ for 15 min at room temperature. The filters were then washed as follows: 1 h in 0.6 M NaCl, 10 mM Hepes, pH 7.0, 2 mM EDTA, and 0.1% $NaDodSO_4$ at 68 °C; 1 h in 2 × SSC and 0.1% $NaDodSO_4$ at 68 °C; 1 h in 2 × SSC at room temperature. The filters were then incubated with RNase (20 μg/mL) in 2 × SSC for 1 h at room temperature, followed by washing twice for 15 min in 2 × SSC at room temperature. The filters were then solubilized in Cellusolve and counted in Aquasol.

Results

Attempts to Identify RNA Transcripts Corresponding to the 5'-End Flanking DNA of the Ovalbumin Gene in Steady-State Nuclear RNA. To examine whether DNA sequences flanking the 5' end are transcribed, we prepared different cloned fragments corresponding to the 5' end of the gene, nick translated them, and used them as probes to assay for the presence of such transcripts in oviduct nuclear RNA. The first probe used was a cloned 4.5-kb *Pst*I fragment, which consists of approximately 75% gene sequence and 27% 5'-flanking DNA sequences (Dugaiczyk et al., 1979; Figure 1). When this 4.5-kb probe was hybridized to an excess of oviduct nuclear RNA, the maximum level of hybridization was ~33%. By the assumption that only one strand of this DNA was transcribed in vivo, a level of 36% hybridization would be predicted for the 4.5-kb fragment if transcription was initiated at or near the beginning of the 5' structural sequence. A 5.5-kb *Eco*RI fragment which consists of approximately 6% gene sequence and 94% 5'-flanking DNA sequences (Dugaiczyk et al., 1979; Figure 1) was used as a hybridization probe to verify the above observation. Only 5% hybridization was obtained (Figure 2), which agreed well with the predicted levels of hybridization (3%) for the 5.5-kb fragment if transcription started near the beginning of the 5' structural sequence. Thus, the above results suggest that transcripts corresponding to DNA sequences flanking the 5' end of the gene are not present in the nuclear RNA.

The 4.5-kb *Pst*I fragment was further digested with *Mbo*II and *Hin*dIII to produce a smaller fragment which consists of 54% gene sequence and 46% 5'-flanking sequence to examine more closely whether the flanking DNA sequences are present as transcripts. This fragment is 0.46 kb in length (Figure 1).

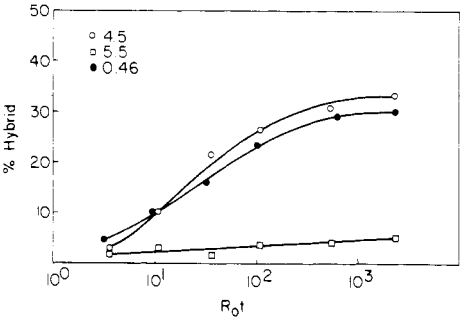

FIGURE 2: Hybridization of oviduct nuclear RNA to ^{3}H-labeled probes from the 5' region of the ovalbumin gene. The probes used were the 4.5-kb *Pst*I fragment (○), the 5.5-kb RI fragment (□), and the 0.46-kb *Mbo*II–*Hin*dIII fragment (●). Hybridization was performed as described under Methods.

Hybridization of the labeled 0.46-kb fragment with an excess of nuclear RNA resulted in protection of 30% of this fragment from digestion with S_1 nuclease (Figure 2). If transcription had initiated at the beginning of the structural sequence, 27% of the fragment would have hybridized. Although the additional hybridization observed (3%) suggests that some of the flanking DNA may be transcribed (~15 nucleotides), we feel that this is within the experimental error of the assay. In this study, the hybridization reaction was carried out to an R_0t value of more than 2×10^3 M s. At this R_0t value, RNA transcripts existing at a concentration of 0.5 molecule/cell should be able to protect 50% of the probe against S_1 nuclease digestion. Therefore, the lack of hybridization at this R_0t value indicated the existence of much less than 0.1 molecule of this flanking sequence per nucleus.

Transcription of the 5' Region of the Ovalbumin Gene in Isolated Nuclei and in Tissue Suspensions of Chick Oviduct. The failure to detect significant amounts of RNA transcripts corresponding to the 5'-end flanking DNA sequences suggests that these sequences either are not transcribed in vivo or are transcribed but rapidly processed, resulting in no detectable accumulation of these sequences in steady-state RNA. RNA was synthesized from isolated nuclei and from oviduct suspension culture in the hope of reducing the effect of processing events to differentiate between these two possibilities. ^{32}P-Labeled RNA was synthesized in oviduct nuclei for 30 min, isolated, and hybridized to a filter containing DNA fragments which had been electrophoresed in agarose gels and transferred

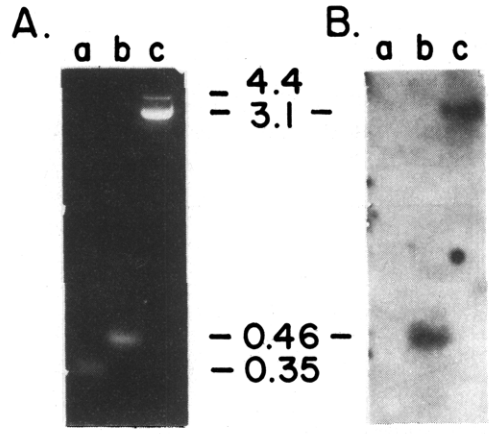

FIGURE 3: Hybridization of in vitro synthesized RNA to probes from the 5′ region of the ovalbumin gene. DNA fragments from the 5′ region of the gene were electrophoresed in an agarose gel. Panel A shows the ethidium bromide stain of the gel, and panel B shows the corresponding autoradiogram after the DNA was transferred from the gel to a nitrocellulose filter and hybridized to [^{32}P]RNA synthesized in nuclei. Lane a contains the 0.35-kb *Mbo*II fragment, lane b the 0.46-kb *Mbo*II–*Hin*dIII fragment, and lane c the 3.1-kb *Hin*dIII–*Pst*I fragment and a minor brand of pBR322 which is 4.4 kb in length.

to a nitrocellulose filter by the method of Southern (1975).

The DNA filter contained the 3.1-kb *Hin*dIII–*Pst*I fragment (which consists entirely of ovalbumin gene sequence), the 0.46-kb *Mbo*II–*Hin*dIII fragment (which consists of 250 nucleotides of the 5′ end of the gene and 210 nucleotides of 5′-flanking DNA), and the 0.35-kb *Mbo*II fragment [which consists entirely of 5′-flanking DNA to the left of the 0.46-kb fragment (Figure 1)]. The ethidium bromide staining pattern for these fragments is shown in Figure 3A. The labeled RNA hybridized to the 3.1- and 0.46-kb fragments but not to the 0.35-kb fragment (Figure 3B). Therefore, transcription of the ovalbumin gene in nuclei appears to mimic that observed in vivo.

The above studies were extended by pulse labeling RNA for shorter periods of time, ranging from 5 to 20 min, to minimize the processing of the in vitro synthesized RNA. The amount of RNA transcribed from the 5′ region of the gene was determined by hybridizing the [^{3}H]RNA to filters containing the 4.5-kb *Pst*I fragment (pOV4.5). We assayed for transcripts of the 5′-flanking DNA by performing the hybridization in the presence of competitor RNA which was synthesized from the 3.2-kb *Hin*dIII fragment. The cloned 3.2-kb *Hin*dIII fragment contains all the ovalbumin sequences in the 4.5-kb *Pst*I fragment except for ~250 nucleotides to the left of the 5′ *Hin*dIII site (Figure 1). If transcription initiates at the beginning of the 5′ structural sequence, $RNA_{3.2}$ should compete out more than 90% of the hybridizable counts. However, if all of the 5′-flanking DNA represented in the 4.5-kb *Pst*I fragment is transcribed, only ~65% competition should occur. It can be seen in Table I that for all labeling periods from 5 to 20 min, ~90% or greater competition was observed in the presence of $RNA_{3.2}$. For control purposes, the labeled RNA was also hybridized to filters containing the 3.2-kb *Hin*dIII fragment (pOV3.2). The number of counts bound to the pOV3.2 filters is similar to that observed for the pOV4.5 filters, as would be expected if the 5′-flanking DNA is not transcribed (Table I). Thus, even with the shortest pulse, no significant amount of 5′-flanking DNA sequences can be detected in the in vitro transcripts.

The results obtained with RNA synthesized in nuclei were confirmed by pulse labeling RNA in an oviduct tissue suspension system developed by McKnight (1978) and modified in our laboratory (M.-J. Tsai, A. C. Ting, J. L. Nordstrom,

Table I: In Vitro Transcription of the 5′ Region of the Ovalbumin Gene[a]

filter	competitor	[^{3}H]RNA hybridized (cpm) after pulse label[b] 5 min	10 min	20 min
RNA Synthesized in Nuclei				
pOV4.5		960	1170	1450
	$RNA_{3.2}$	123	26	0
pOV3.2		1190	993	1350
	$RNA_{3.2}$	41		135

filter	competitor	[^{3}H]RNA hybridized (cpm) after pulse label[c] 5 min	10 min
RNA Synthesized in Tissue Suspension System			
pOV4.5		170	272
	$RNA_{3.2}$	32	27
pOV3.2		182	269
	$RNA_{3.2}$	22	33

[a] The background of the control filter was 34 ± 3 cpm, and the standard deviation of the hybridizable counts from an average of 10 sets of experimental data was ±6%. [b] Input [^{3}H]RNA = 1.4 × 10^6 cpm. [c] Input [^{3}H]RNA = 6 × 10^6 cpm.

and B. W. O'Malley, unpublished experiments). Although the number of hybridizable counts was less for RNA synthesized in this system than in isolated nuclei, the results were qualitatively similar (Table I). Thus, the failure to detect pulse-labeled RNA corresponding to the 5′-flanking DNA argues against transcription initiating farther upstream from the 5′ structural sequence, followed by rapid processing to form the molecules observed in steady-state nuclear RNA.

A Search for Transcripts Corresponding to DNA Flanking the 3′ End of the Ovalbumin Gene. The 3′ portion of the ovalbumin gene is located in a closed 9.2-kb *Eco*RI fragment (Dugaiczyk et al., 1979; Figure 1). This fragment contains 2150 nucleotides of the 3′ end of the ovalbumin gene and ~7050 nucleotides of 3′-flanking sequence. Initially, we wanted to determine how much of the 9.2-kb fragment was transcribed in vivo. The 9.2-kb fragment was purified by electrophoresis in agarose gels and labeled by nick translation. The labeled fragment was then hybridized to an excess of oviduct nuclear RNA (Figure 4A). Approximately 14% hybridization was observed. Ovalbumin gene sequences comprise ~23% of the 9.2-kb fragment. If we assume that transcription of the ovalbumin gene terminates at the end of its structural sequence and that only one strand of the DNA is transcribed in vivo, we would expect ~12% of the 9.2-kb fragment to hybridize to oviduct nuclear RNA. Therefore, the results suggest that termination of transcription of the ovalbumin gene appears to occur near the end of the 3′ structural sequence.

The 9.2-kb fragment can be digested with *Hin*dIII to form smaller fragments that are 2.7, 3.2, 1.7, and 1.7 kb in length (Figure 1). The 2.7- and 3.2-kb fragments and a mixture of the 1.7-kb fragments were purified by agarose gel electrophoresis and labeled by nick translation (the 1.7-kb fragments electrophoresed as a single band, and no attempt was made to separate these fragments). These labeled fragments were then hybridized to an excess of oviduct nuclear RNA (Figure 4B). Structural sequences comprise ~83% of the 2.7-kb fragment (2150 nucleotides). If transcription terminates at the end of the structural sequence of the gene and if only one strand of the DNA is transcribed, then ~42% hybridization would be expected with the 2.7-kb probe. As shown in Figure 4B, the 2.7-kb fragment hybridized to ~42%, in good

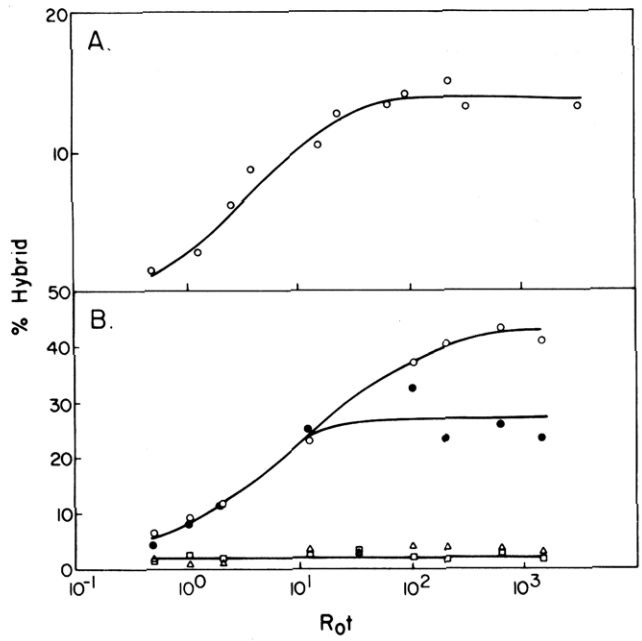

FIGURE 4: Hybridization of oviduct nuclear RNA to ^{3}H-labeled probes from the 3′ region of the ovalbumin gene. The nuclear RNA was hybridized to (A) the 9.5-kb RI fragment and (B) the 2.7-kb RI–*Hin*dIII fragment (○), the 3.2-kb *Hin*dIII fragment (□), the 1.7-kb *Hin*dIII fragments (△), and the 0.25-kb *Hin*fI–*Hph*I fragment (●).

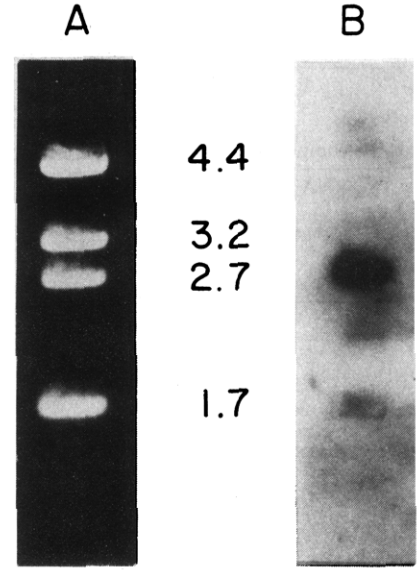

FIGURE 5: Hybridization of in vitro synthesized RNA to probes from the 3′ region of the ovalbumin gene. The cloned 9.2-kb RI fragment was cleaved with restriction endonuclease *Hin*dIII and electrophoresed in an agarose gel. Lane A shows the ethidium bromide stain of fragments that are 4.4 (pBR322), 3.2, 2.7, and 1.7 kb in length, and lane B shows the corresponding autoradiogram after the DNA was transferred from the gel to a nitrocellulose filter and hybridized to [^{32}P]RNA synthesized in nuclei.

agreement with the predicted value. In contrast, no hybridization was observed with the 3.2- and 1.7-kb fragments, as predicted from the results obtained with the 9.2-kb fragment. Therefore, no detectable transcripts corresponding to these 3′-flanking regions are present in oviduct nuclear RNA.

Further studies on transcription of the 3′-end flanking sequences were carried out by using a smaller 0.25-kb fragment which contains 127 nucleotides of structural sequence and 125 nucleotides of 3′-flanking DNA sequence (Figure 1). This fragment was obtained by digesting the 2.7-kb fragment with *Hin*fI and *Hph*I. The 0.25-kb fragment hybridized to ~25% in the presence of an excess of oviduct nuclear RNA (Figure 4B). This is the value expected if transcription terminates at the end of the structural sequences and only one strand of the DNA is transcribed. Thus, even assuming a maximum of 5% error in the hybridization analyses, no more than 15 nucleotides past the end of the structural sequences appear to be transcribed.

It should be noted that the profile of the hybridization curve obtained with the 2.7-kb probe is quite broad. This was not unexpected since the ovalbumin sequences in the 2.7-kb fragment correspond to 1100 nucleotides of intervening sequence and 1050 nucleotides of structural sequence (Figure 1) and we have previously shown that the concentration of transcripts corresponding to structural sequences was approximately 10-fold greater than that corresponding to intervening sequences (Roop et al., 1978). Since the 0.25-kb fragment contains only structural sequences and no intervening sequences, its hybridization curve has a sharp transition and occurs over an R_0t range expected for structural sequences. As expected, the profile of the hybridization curve obtained with the 9.2-kb fragment is similar to that observed for the 2.7-kb probe (parts A and B of Figure 4).

In Vitro Transcription of the 3′ Region of the Ovalbumin Gene. Labeled RNA synthesized in nuclei was assayed to determine if transcripts corresponding to the 3′-flanking sequences were synthesized in this in vitro system. The 9.2-kb fragment was digested with *Hin*dIII, electrophoresed in agarose gels, and transferred to a nitrocellulose filter. The ethidium bromide staining pattern is shown in lane A of Figure 5. The ^{32}P-labeled RNA was hybridized to the DNA filter and the pattern visualized by radioautography (Figure 5, lane B). The labeled RNA only hybridized to the 2.7-kb fragment and not to the 3.2- and 1.7-kb fragments. Therefore, termination of transcription of the ovalbumin gene in nuclei occurs within the 2.7-kb fragment and no detectable transcript existed for the flanking DNA.

RNA was also labeled for shorter times and hybridized to filters containing the 9.2-kb RI fragment. RNA synthesized from the 2.7-kb RI–*Hin*dIII fragment was used as a competitor to determine if the flanking DNA to the right of the 2.7-kb fragment was transcribed. If transcription terminates near the end of the structural sequence, then the pulse-labeled RNA should not hybridize to the pOV9.2 filters in the presence of competitor $RNA_{2.7}$. More than 94% of the radioactivity hybridized to the pOV9.2 filters was competable by $RNA_{2.7}$ (Table II). The labeled RNA was also hybridized to control filters containing the 2.7-kb RI–*Hin*dIII fragment (pOV2.7). The number of counts bound to these filters was similar to that observed for the pOV9.2 filters and also indicates that transcription terminates within the 2.7-kb fragment (Table II). Qualitatively similar results were also obtained with RNA pulse labeled in the oviduct tissue suspension system (Table II). Since no pulse-labeled RNA was detected which corresponds to the 3′-flanking DNA to the right of the 2.7-kb fragment, transcription does not appear to continue downstream past this fragment with subsequent processing to form the molecules observed in steady-state nuclear RNA.

Discussion

Recently, we have demonstrated that multiple species of RNA which contain both structural and intervening sequences of the ovalbumin gene are present in oviduct nuclei (Roop et al., 1978). The largest ovalbumin RNA molecules in steady-state RNA and in pulse-labeled RNA in oviduct tissue are ~7.8 kb in size, which is similar to the size of the ovalbumin natural gene (M.-J. Tsai, A. C. Ting, J. L. Nordstrom,

Table II: In Vitro Transcription of the 3′ Region of the Ovalbumin Gene

filter	competitor	[^{3}H] RNA hybridized (cpm) after pulse label[a] 5 min	10 min	20 min
RNA Synthesized in Nuclei				
pOV9.2		578	732	1300
	$RNA_{2.7}$	35	34	0
pOV2.7		844	867	1220
	$RNA_{2.7}$	6		0

filter	competitor	[^{3}H] RNA hybridized (cpm) after pulse label[b] 5 min	10 min
RNA Synthesized in Tissue Suspension System			
pOV9.2		107	163
	$RNA_{2.7}$	10	15
pOV2.7		126	169
	$RNA_{2.7}$	23	0

[a] Input [^{3}H] RNA = 1.4×10^6 cpm. [b] Input [^{3}H] RNA = 6×10^6 cpm.

and B. W. O'Malley, unpublished experiments). By use of a modification of the S_1 nuclease mapping technique of Berk & Sharp (1977), the 5′ and 3′ ends of the transcripts of the ovalbumin gene have been mapped with respect to the physical map of the cloned DNA and these termini are coincident with the beginning and end of the structural sequence of the natural gene (Roop et al., 1980).

S_1 nuclease mapping can measure accurately the location of the 5′ and 3′ ends of the transcripts; however, if the transcripts are present in very low concentration, its termini may not be scored by the mapping technique. For this reason, R_0t analysis, a much more sensitive method, is used in the present study. The hybridization reactions were carried out to an R_0t value greater than 3×10^3 M s, which has previously been shown to allow detection of less than 0.1 molecule of $mRNA_{ov}$ per cell nucleus in chick oviduct (Roop et al., 1978). Even under these conditions, we have not been able to detect any sequences corresponding to flanking regions spanning 5 kb to the left of the 5′ end and 7 kb to the right of the 3′ end. Therefore, these sequences either are not transcribed or are transcribed, processed, and subsequently degraded so thoroughly that fewer than 0.1 molecule/cell nucleus can be found in the steady-state nuclear RNA. Harris et al. (1975) have estimated that at least 20–30 molecules of polymerase are engaged in RNA synthesis of the ovalbumin gene at a given time. Thus, the RNA used in the hybridization must contain more than 20–30 molecules of nascent RNA chains per nuclear equivalent of RNA. Thus, if sequences flanking the 5′ and 3′ ends of the ovalbumin are transcribed but degraded before completion of the chain, this requires that the rate of degradation of such transcripts be equal to its rate of synthesis.

Pulse-labeled RNAs in isolated nuclei were used to measure the existence of flanking RNA transcripts to minimize the effect of processing and degradation. No significant difference can be demonstrated during different labeling periods. Even at the shortest labeling period (5 min), we have been unable to detect transcripts of the sequences flanking the 5′ and 3′ ends of the gene. In addition, pulse-labeled RNA isolated from oviduct tissue suspensions, which closely resemble the in vivo conditions, did not contain any flanking DNA sequence transcripts. These results are consistent with the result that no transcripts larger than 7.8 kb can be identified in RNA pulse labeled in the oviduct suspension system (M.-J. Tsai, A. C. Ting, J. L. Nordstrom, and B. W. O'Malley, unpublished experiments). These results support again the observations made with steady-state nuclear RNA that initiation and termination transcription of the ovalbumin gene begin and end at a point which is very near the beginning and the end of the structural sequences. However, we can not exclude the unlikely possibility that DNA sequences flanking the natural gene are transcribed and immediately degraded within the shortest labeling period of our assays.

Recent studies by Ziff & Evans (1978) and Manley et al. (1979) have demonstrated that in the case of the adenovirus late mRNA, the capped 5′ terminus is the initiation site of transcription. If the 15S β-globin precursor proves to be the primary transcript, the initiating residue of the 5′ terminus of the 15S precursor (Weaver et al., 1979) is conserved in mRNA as the capped 5′ terminus (Curtis et al., 1977). Since we are not able to detect transcripts of the 5′- and 3′-flanking sequences of the gene, it is very likely that the largest nuclear precursor (7.8 kb) to ovalbumin mRNA is the initial transcript of the ovalbumin gene.

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Interactions of Bleomycin Analogues with Deoxyribonucleic Acid and Metal Ions Studied by Fluorescence Quenching†

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ABSTRACT: Fluorescence quenching has been used to study the interactions of DNA, in the presence and absence of metal ions, with various bleomycin (BLM) analogues, which include the active BLM A_2, BLM B_1', talisomycin A (TLM A), desamido-BLM A_2, the three less active BLM A_2 analogues with a substitution at the α-amino group of the β-aminoalanine, and the two inactive TLM fragments (W_a and W_b). All drugs showed a nonionic type DNA-induced fluorescence quenching of 20–30% but varied in the extent of the ionic type of quenching as follows: TLM A, W_α, and W_b, 50–60%; BLM A_2, 20–25%; BLM B_1' and desamido-BLM A_2, <10%; the three α-amino-substituted BLM A_2 analogues, 0%. Thus, there is no apparent correlation of the overall DNA breakage activity of drugs with the extent of either type of quenching. Thus, DNA–drug interactions as seen by quenching effects are not sufficient for breakage activity. However, the modification of the α-amino group completely eliminated the ionic quenching and reduced the nonionic type. The removal of the terminal amide group of the β-aminoalanine as in desamido-BLM A_2 markedly reduced the ionic quenching, whereas an increase in the positive charge of the C-terminal amine enhanced the ionic quenching. Thus, both the β-aminoalanine and the C-terminal amine may be essential for the ionic type of quenching effect, which may be associated with the DNA double-strand breakage activity of BLM since such activity was very low in desamido-BLM A_2 in contrast to BLM A_2. The binding analysis indicated that at high ionic strength, the removal of the amide or the loss of the positive charge in the terminal amine minimally affected the binding constant but reduced the number of available binding sites by 50%. These residual sites are nonionic in nature. The overall quenching effect showed a base and sequence preference consistent with the reported GC, GT, and TA specificity of the DNA breakage. The quenching effects of DNA and Fe(II) or Cu(II) were additive. β-Mercaptoethanol, which showed a differential effect on Fe(II)- and Cu(II)-induced quenching, had little effect on the DNA-induced quenching.

Bleomycins are a family of glycopeptides, some of which have been clinically used to treat certain human tumors (Umezawa, 1976; Crooke & Bradner, 1976; Muller & Zahn, 1977; Goldberg et al., 1977). The antitumor activity of the bleomycins is thought to be related to the ability of bleomycins to induce single- and double-strand breakage of DNA molecules (Suzuki et al., 1969; Haidle, 1971; Muller et al., 1972; Takeshita et al., 1974).

It has been reported that DNA quenched the fluorescence of bleomycin A_2 (Chien et al., 1977; Strong & Crooke, 1978a) and of talisomycin (Strong & Crooke, 1978a,b). In a previous report (Huang et al., 1979), we have demonstrated that the quenching effects of Fe(II) and Cu(II) ions on the fluorescence of various bleomycin analogues were related to the ability of bleomycin analogues to induce DNA strand breakage. The purpose of the present study was to investigate the interactions of bleomycin analogues with DNA in the presence or absence of externally added metal ions and the relationship between these interactions and the ability of the bleomycin analogues to induce DNA breakage.

Materials and Methods

Chemicals. The following bleomycin analogues and hydrolytic fragments were obtained from Bristol Laboratories: bleomycin (BLM)[1] A_2, BLM B_1', talisomycin (TLM) A, methylsulfonamido-BLM A_2, benzylsulfonamido-BLM A_2, dansylsulfonamido-BLM A_2, and TLM fragments W_a and W_b. The chemistry and biology of the semisynthetic compounds are subjects of a separate report. Desamido-BLM A_2 was a generous gift from Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. PM-2 DNA was isolated according to the procedure of Strong & Hewitt (1975). For fluorescence studies, each PM-2 DNA preparation was dialyzed against the Tris-HCl buffer used in fluorescence assays to reduce the NaCl concentration. Calf thymus DNA, *Micrococcus lysodeikticus* DNA, *Clostridium perfringens* DNA, and yeast tRNA were obtained from Sigma Co. (St. Louis, MO). Chloride salts of Fe(II) and Cu(II) were obtained from Mallinckrodt Co. (St. Louis, MO). Synthetic polynucleotides were purchased from P-L Biochemicals Co. (Milwaukee, WI).

Fluorescence Measurements. Fluorescence was measured with an Amico-Bowman spectrophotofluorometer in a 1-mL cuvette. The reaction buffer (1 mL) contained 2.5 mM

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[1] Abbreviations used: BLM, bleomycin; Na_2EDTA, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; TLM, talisomycin.

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