Deoxyribonuclease I Sensitivity of the Nontranscribed Sequences Flanking the 5′ and 3′ Ends of the Ovomucoid Gene and the Ovalbumin and Its Related X and Y Genes in Hen Oviduct Nuclei†

George M. Lawson,* Ming-Jer Tsai, and Bert W. O'Malley

ABSTRACT: When hen oviduct nuclei were digested with pancreatic DNase I under conditions known to preferentially degrade transcriptionally active genes (i.e., 14–21% of the DNA rendered perchloric acid soluble), the transcribed DNA sequences (including both structural and intervening sequence DNA) of the ovomucoid gene (5.6 kb) and the ovalbumin gene (7.6 kb) were depleted in concentrations approximately 2.5-fold compared to control DNA preparations not subjected to in situ nucleolysis as determined by standard C_0t analysis. The immediately adjacent nontranscribed sequences including at least 7.8 and 5.8 kb of DNA flanking respectively the 5′ and 3′ ends of the ovomucoid gene and at least 5.2 and 7.1 kb flanking the 5′ and 3′ ends of the ovalbumin gene also exhibited a DNase I sensitivity indistinguishable from that observed for the transcribed regions. In contrast, these same ovomucoid- and ovalbumin-specific sequences were resistant to DNase I in spleen, liver, and erythrocyte nuclei, while a sequence including the entire natural β-globin gene was resistant in oviduct, spleen, and liver nuclei but sensitive in erythrocyte nuclei. The hormonally modulated X and Y genes, which are closely linked to the ovalbumin gene but transcribed at only 1 and 6% that of ovalbumin, also exhibited a DNase I sensitivity in oviduct nuclei comparable to that of ovalbumin and ovomucoid. Moreover, the sensitive region includes at least 8.4 kb of DNA flanking the 5′ end of the X gene. These data indicate that those aspects of chromatin structure which confer DNase I sensitivity to expressible genes are not necessarily confined within the transcription domain and further suggest that, at least for some genes, a transition from a DNase I sensitive to an insensitive state occurring at the junctions of the gene and its flanking sequences is apparently not involved in delineating the boundaries of the transcription unit. Such organization can lead to polygenic domains in which the transcribed structural and intervening sequence DNA as well as the nontranscribed intergenic spacer exists in a DNase I sensitive conformation. The ovalbumin domain includes a minimum of three genes contained in at least 54 kb of DNase I sensitive chromatin.

The major egg white proteins, ovalbumin and ovomucoid, are synthesized in large amounts in the oviducts of laying hens or estrogen-primed chicks (O'Malley et al., 1969; Rhoads et al., 1973; Palmiter, 1973; Hynes et al., 1977; Tsai et al., 1978). The successful cloning of defined fragments of genomic DNA containing the genes which code for these proteins has provided novel insights into gene structure and expression. In the case of the ovalbumin gene (Woo et al., 1978; Dugaiczyk et al., 1979; Gannon et al., 1979; O'Hare et al., 1979) and the ovomucoid gene (Catterall et al., 1979; Lai et al., 1979), the DNA sequences which code for the mature messenger RNA are not collinear but are interrupted by seven intervening sequences of as yet undefined function. Studies on the expression of these genes indicate that each is transcribed in its entirety to generate a primary transcript which contains both structural and intervening sequences; these transcripts are ultimately processed to the mature messenger RNA by removal of the intervening sequences through a series of excision and ligation events (Roop et al., 1978; Nordstrom et al., 1979; M.-J. Tsai et al., unpublished results). The initiation and termination sites for transcription have been precisely localized by mapping the 5′ and 3′ ends of the precursors and found to coincide in both cases with the 5′ and 3′ termini of the mature messenger RNA (Roop et al., 1980; Tsai et al., 1980).

The specific mechanisms which regulate the transcriptional activity of these genes in vivo remain obscure. Evidence from several sources suggests that genes having transcriptional potential exist in an altered conformation in the chromatin which renders them more susceptible to attack by exogenous nucleases as compared to genes that are never expressed (Weintraub & Groudine, 1976; Garel & Axel, 1976; Panet & Cedar, 1977; Levy & Dixon, 1977; Palmiter et al., 1977). It now appears that the sequestration of a gene, or more precisely the nucleosomes which comprise that gene, into an "active" conformation may be a prerequisite to the transcription of that gene. Moreover, in at least one system it has been reported that the active conformation extends only over that region of a gene which is transcribed, suggesting that a switch from an active to an inactive conformation occurring at the extremities of a gene may play a role in delineating the boundaries of the transcription unit (Flint & Weintraub, 1977).

The studies presented here were conducted to determine if similar structural transitions occur at the junctions which separate the transcribed sequences of the ovomucoid and ovalbumin genes from their 5′ and 3′ nontranscribed flanking sequences. Hen oviduct nuclei were treated in situ with pancreatic DNase I under conditions favoring the preferential digestion of active genes, and the relative nucleolytic sensitivity of transcribed and nontranscribed flanking sequences was assayed by hybridizing the DNase I resistant DNA against probes specific to the regions of interest. In addition, the DNase I sensitivities of the hormonally responsive X and Y genes which are closely linked to the ovalbumin gene but expressed respectively at levels of only 1 and 6% that of ovalbumin (Royal et al., 1979; Colbert et al., in press) were also investigated.

† From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030. *Received March 21, 1980.* This work was supported by National Institutes of Health Grant HD-08188 and the Baylor Center for Reproductive Biology, HD-07495. G.M.L. was the recipient of National Institutes of Health Postdoctoral Fellowship GM-06391-01.

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Materials and Methods

Isolation of Nuclei. (*A*) *Hen Oviduct.* Nuclei were isolated from the magnum portion of hen oviduct (ca. 100 g of oviduct wet weight per preparation) as previously described (Tsai et al., 1975) with the following modifications. The oviduct homogenate, prepared in TKM buffer (0.05 M Tris-HCl, 0.02 M KCl, and 5 mM $MgCl_2$, pH 7.5) containing 0.5 M sucrose, was centrifuged for 10 min at 3000*g*. The crude nuclear pellet was washed twice by resuspension and centrifugation (10 min at 3000*g*) in the same buffer also containing 0.5% Triton X-100. The pellet recovered from the second detergent wash was resuspended in the same buffer, and the suspension was again centrifuged for 10 min at 3000*g* after being layered over a cushion of 0.88 M sucrose and TKM buffer containing no detergent. The pelleted nuclei were resuspended in 2.0 M sucrose and TKM buffer and centrifuged for 40 min at 12000*g*. Final resuspension of the purified nuclei was in a buffer containing 0.01 M Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$ (pH 7.5), and 0.35 M sucrose.

(*B*) *Chick Spleen and Chick Liver.* Spleen nuclei were isolated by the above procedure without modification. Liver nuclei were isolated by the above procedure except that the crude nuclear pellet was washed 2 additional times in 0.5 M sucrose and TKM buffer containing 0.5% Triton X-100 prior to centrifugation through the 0.88 M sucrose and TKM cushion.

(*C*) *Hen Erythrocyte.* Packed blood cells (obtained by cardiac puncture) were washed twice in 0.5 M sucrose and TKM buffer containing heparin (10 units/mL), once in 0.5 M sucrose and TKM buffer alone, and then lysed in 0.5 M sucrose and TKM buffer containing 0.5% Triton X-100. Erythrocyte nuclei were subsequently purified as described above for oviduct nuclei.

Nuclei from each source were quantitated after appropriate dilution of an aliquot into 1 M NaOH by measuring the absorbance at 260 nm (corrected for a slight light-scattering contribution by subtraction of the absorbance at 320 nm) and applying the expression: 1 mg/mL DNA = 24 A_{260} units. Values using this method were found to agree within 10–15% when compared to values obtained following prior removal of sucrose, complete hydrolysis in hot perchloric acid, and subsequent DNA quantitation by diphenylamine assay (Burton, 1956). The final suspension was at a DNA concentration of 1–1.5 mg/mL and nuclei were either digested with DNase I or used as a source of control, undigested DNA as described below. Freshly prepared nuclei were used in all experiments.

DNase I Treatment of Nuclei and Isolation of DNA Digestion Fragments. Nuclei were digested at 37 °C with 40 μg/mL pancreatic DNase I (Worthington, 2029 units/mL) until 14–21% of the DNA was rendered soluble in cold perchloric acid (Garel & Axel, 1976). The time required to obtain the desired extent of digestion varied slightly with the nuclear preparation but generally was about 5 min. Digestion was terminated by the addition of EDTA, NaCl, sodium dodecyl sulfate ($NaDodSO_4$), and proteinase K (EM Biochemicals) to final concentrations of 0.02 M, 0.1 M, 0.2%, and 50 μg/mL, respectively, followed by incubation for 1 h at 37 °C. Nucleic acid was extracted by addition of an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1 v/v). The aqueous phase was removed and extracted a second time with an equal volume of chloroform–isoamyl alcohol (24:1 v/v). Nucleic acid was precipitated from the aqueous phase by addition of 2 volumes of ethanol (16 h at −20 °C). The pellet was resuspended in 0.02 M Tris-HCl, 0.1 M NaCl, and 1 mM EDTA (pH 7.5) and redigested with proteinase K (20 μg/mL) at 37 °C for 30 min. Sodium hydroxide was then added to a concentration of 0.3 M, and the preparation was incubated at 68 °C for 45 min to hydrolyze RNA. Hydrolysis was terminated by neutralization with HCl, and the DNA was chromatographed on a column of Sephadex G-50 containing a plug of Chelex 100 (Bio-Rad); the column was eluted in 0.01 M Tris-HCl, 0.01 M NaCl, and 1 mM EDTA (pH 7.5). Fractions comprising the excluded volume were made 0.1 M in NaCl and pooled, and the DNA was precipitated by the addition of 2 volumes of ethanol. The purified DNA was resuspended in deionized, distilled water at a concentration of 15 mg/mL and stored at 4 °C.

Preparation of Control DNA. Control DNA from each tissue was prepared from the same batch of nuclei which, in the initial stages, were treated as described above except that they were not subjected to DNase I digestion. The deproteinized nucleic acid pellet from the first ethanol precipitation was resuspended in 0.02 M Tris-HCl, 0.1 M NaCl, and 3 mM $MgCl_2$ (pH 7.5) at a concentration of approximately 5 mg/mL and "sheared" by digestion at 37 °C with pancreatic DNase I (15 μg/mL) to generate a DNA fragment population comparable in size to that obtained from DNase I digested nuclei (see below). Digestion was quenched by the addition of EDTA, $NaDodSO_4$, and proteinase K to final concentrations of 0.02 M, 0.2%, and 20 μg/mL, respectively, followed by incubation at 37 °C for 30 min. Hydrolysis of RNA and gel filtration over Sephadex G-50/Chelex 100 were performed as described above for the isolation of DNA from DNase I digested nuclei.

Polyacrylamide Gel Electrophoresis of DNA Fragments. Size estimates of DNA digestion fragments were obtained by electrophoresis under denaturing conditions in 10% polyacrylamide–7 M urea slab gels (12 × 15 × 0.1 cm) as described in Maniatis et al. (1975). DNA samples were dissolved in 99% formamide and incubated at 95 °C just prior to loading. Electrophoresis was at 20 mA for 1.5 h. Gels were stained with 0.001% ethidium bromide for 30 min and photographed under ultraviolet illumination.

Preparation and Labeling of DNA Restriction Fragments Comprising Discrete Regions of the Ovomucoid and Ovalbumin Genes and Their Contiguous Flanking Sequences. Various clones which contain portions of the ovomucoid and ovalbumin genes and their 5′ and 3′ flanking sequences have been isolated from total chick DNA in our laboratory by using standard cloning techniques (Dugaiczyk et al., 1979; Woo et al., 1978; Catterall et al., 1979). Other clones were isolated from a chicken gene library (obtained from Drs. Engel, Dodgson, Axel, and Maniatis) prepared by using Charon *4A* λ DNA vector and chicken DNA that was partially digested by *Hae*III and *Alu*I and ligated to synthetic *Eco*RI linkers (Maniatis et al., 1975). The isolation and partial characterization of clones containing ovomucoid and ovalbumin DNA obtained from this library, as well as clones containing DNA from the X and Y genes, including sequences which flank the 5′ end of the X gene, have also been described (Dugaiczyk et al., 1979; Lai et al., 1979; Colbert et al., in press). A globin clone containing a 6.1-kb insert was selected from an RPC fraction of *Eco*RI-digested chick DNA by screening with a cDNA probe prepared against chicken globin messenger RNA. Subsequent characterization has shown this fragment to be identical with the 6.1-kb *Eco*RI fragment of Ginder et al. (1979) which contains the entire adult β-globin gene as well as the 5′ half of an adjacent β-globin-like gene (Day et al., in press).

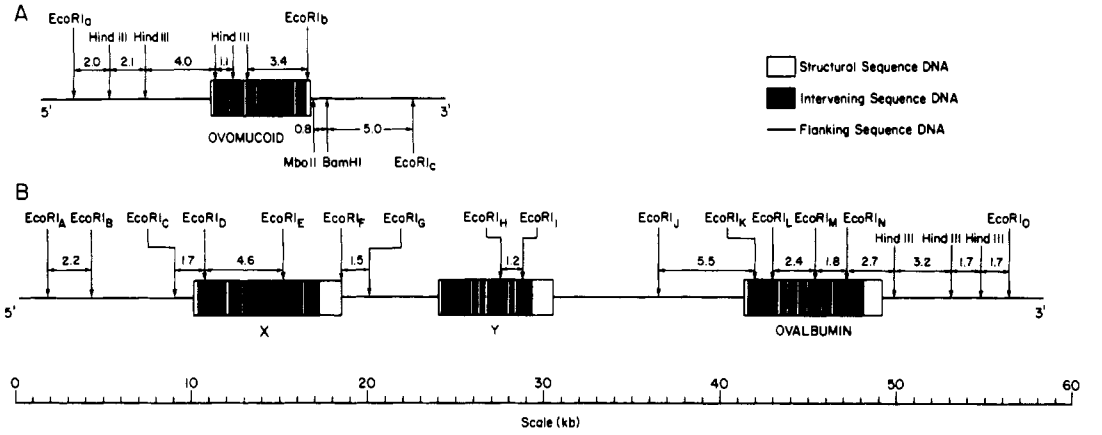

FIGURE 1: Gene maps showing the specific DNA sequences (i.e., restriction fragments) probed for DNase I sensitivity. (A) The natural ovomucoid gene and its 5′ and 3′ nontranscribed flanking DNA. (B) The natural ovalbumin gene and its 5′ and 3′ nontranscribed flanking DNA including the adjacent X and Y genes. Restriction fragments are identified by their length in kilobases. Both maps are drawn to the same scale. Positions of interevening and structural sequences for the X and Y genes have been taken from Royal et al. (1979) and Colbert et al. (in press). Restriction sites *Eco*RI$_A$ and *Eco*RI$_J$ were created artificially during formation of the chick recombinant library; all others occur naturally in the genomic DNA.

The desired restriction fragments were isolated by preparative agarose gel electrophoresis (Roop et al., 1980) following digestion of purified cloned DNA with the appropriate restriction endonuclease(s). The size in kilobases and the relative location of the various restriction fragments used as hybridization probes to specific regions of the ovomucoid and ovalbumin genes, their flanking sequences, and the X and Y genes are shown in the restriction maps of Figure 1 and will be discussed in more detail below. Isolated restriction fragments were labeled by nick translation in the presence of [^{3}H]dCTP and [^{3}H]dTTP (25.6 and 53 Ci/mmol, respectively) by using the procedure of Mackey et al. (1977) as modified by Roop et al. (1978). Specific activities of (2–5) $\times 10^7$ cpm/μg of DNA were routinely obtained. Initial experiments (Figures 3–6) were conducted with probes recovered from the excluded fractions following gel filtration over Sephadex G-50. In subsequent experiments (Figures 7 and 8), nick-translated probes were heat denatured in alkalai, chromatographed over Bio-Gel A1.5M columns, and recovered from the early eluting fractions. The longer probes, which appeared more stable to prolonged incubation, annealed to any given driver DNA preparation with enhanced kinetics (Chamberlin et al., 1978) but maintained the same relative difference in kinetics of annealing to different driver DNA preparations as was observed with the unsized probes.

Detection of Specific DNA Sequences by Liquid Hybridization. Hybridizations were performed in sealed conical glass vials (Kontes) which contained the following components in a final volume of 50 μL: 0.01 M Hepes, 0.6 M NaCl, 0.002 M EDTA (pH 7.0), 7.5–10 mg/mL unlabeled driver DNA obtained from DNase I digested or control nuclei, and 600–800 cpm of nick-translated DNA probe. The ratio of unlabeled DNA to [^{3}H]DNA was at least 10^7 in each analysis. Samples were denatured at 100 °C for 5 min and incubated at 68 °C for various time intervals ranging from 10 min to 40 h. Following hybridization, samples were treated with S1 nuclease (Miles, 4800 units/vial) at 37 °C and nuclease resistant, trichloroacetic acid (Cl_3AcOH) precipitable radioactivity was determined as previously described (Harris et al., 1976). A contribution to S1 nuclease counts resulting from reannealing of complementary strands of the nick-translated probes was monitored by preparation and parallel incubation of vials in which unlabeled chick driver DNA was replaced by an identical concentration of yeast RNA. All vials were corrected for this effect which under the conditions described above rarely exceeded 15% of the total S1 nuclease resistant radioactivity. Equivalent C_0t values (i.e., those that would obtain at 0.18 M Na^+) have been plotted (Britten et al., 1974).

Results

Digestion of Hen Oviduct Nuclei with DNase I and Preparation of DNA for Hybridization Analysis. It has been reported previously that structural regions of the ovalbumin gene, as assayed with a cDNA probe prepared against mature ovalbumin messenger RNA, are preferentially attacked in oviduct nuclei by DNase I (Garel & Axel, 1976). We were interested in whether this nucleolytic sensitivity is confined only to that domain of a gene which is transcribed or if the sensitivity also extends into the 5′ and 3′ nontranscribed regions which flank the gene. To this end, we digested isolated hen oviduct nuclei in situ with pancreatic DNase I. Electrophoretic analysis of the resulting DNA digestion fragments revealed the characteristic 10-nucleotide repeat pattern indicative of the organization of bulk genomic DNA into nucleosomes (Noll, 1974) as shown in Figure 2 (lane 1). In order to determine the relative nucleolytic sensitivity of DNA sequences specific to the regions of interest, we wished to compare the kinetics of annealing when the desired tracer sequences were hybridized against preparations of DNase I resistant DNA as well as to control DNA extracted from nuclei not subject to nucleolysis. Since the rate of annealing is dependent upon fragment length, it was of concern that variations in mean fragment size between control and DNase I resistant DNA preparations might contribute to relative variations in hybridization rates unrelated to the actual concentration of the sequences in question (Chamberlin et al., 1978).

The following approach was taken to eliminate this possibility. DNA was isolated from undigested nuclei and, after deproteinization, was sheared by digestion with DNase I to generate a population of DNA fragments having an overall size distribution roughly comparable to that obtained by in situ digestion of a separate batch of nuclei taken from the same preparation. The control and experimental DNA preparations were further purified for use in hybridization studies as described above. The electrophoretic profile of a typical control DNA preparation, along with its companion experimental preparation, is shown in Figure 2 (lane 2). As expected, the control preparation exhibits no discrete electrophoretic bands, since cleavage by DNase I occurs essentially at random on free DNA. Moreover, those factors conferring DNase I sensitivity

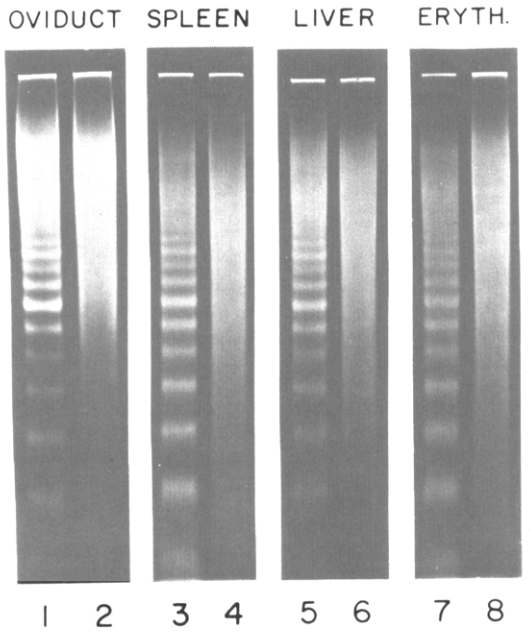

FIGURE 2: Size comparison of typical driver DNA preparations isolated from nuclei derived from different tissues. DNA was electrophoresed under denaturing conditions in a 10% polyacrylamide–7 M urea slab gel. (Lanes 1, 3, 5, and 7) DNA isolated from nuclei after digestion in situ with DNase I. In all cases the extent of digestion was within the range of 14–21% of the DNA rendered soluble in cold perchloric acid. (Lanes 2, 4, 6, and 8) Control DNA isolated from undigested nuclei and sheared with DNase I after deproteionization as described under Materials and Methods. The control and in situ digested DNA preparations of a given pair were always isolated from the same batch of nuclei. Tissue of origin for each pair is designated in the figure. The oviduct pair is one of five separate preparations used in these studies.

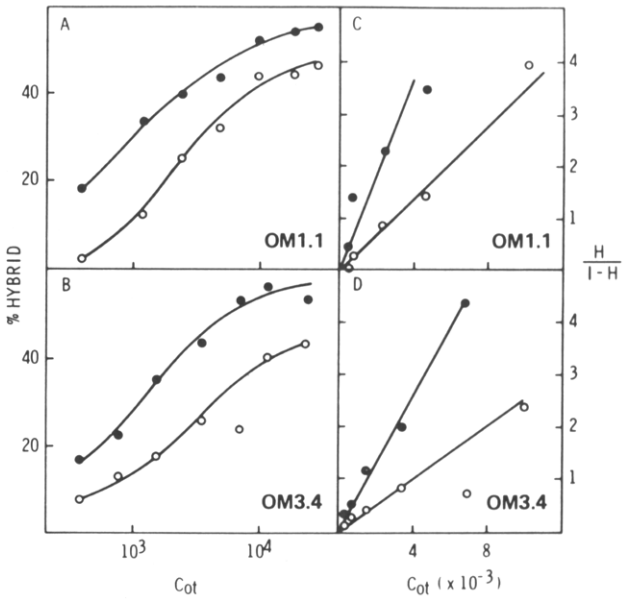

FIGURE 3: DNase I sensitivity of transcribed sequences of the natural ovomucoid gene in oviduct nuclei. Ovomucoid gene specific fragments OM1.1 and OM3.4 were labeled by nick translation and hybridized either to DNA isolated from nuclei following digestion with DNase I or to control DNA prepared from nuclei not subjected to in situ nucleolysis. (A and B) Kinetics of annealing of OM1.1 and OM3.4, respectively, to DNase I digested (○) or control DNA (●) as determined by standard C_0t analysis. Driver DNA concentrations were identical in both cases (10 mg/mL), and hybrid formation was assayed by resistance to S1 nuclease. A small contribution to S1 nuclease resistance resulting from self-annealing of the probes was monitored during the course of hybridization, and the data have been corrected accordingly. (C and D) Transformation of the annealing data for OM1.1 and OM3.4, respectively, to facilitate estimation of the specific sequences in question in the DNase I digested and control DNA preparations. By the assumption of ideal second-order kinetics of reassociation, $H/(1 - H)$ is proportional to C_0t where H represents the fraction of probe existing as hybrid relative to the maximum hybridization observed.

to active genes in chromatin have been shown to reside in the protein moiety (Weintraub & Groudine, 1976; Weisbrod & Weintraub, 1979) so that enzymatic shearing of deproteinized DNA would not be expected to effect a preferential depletion of specific sequences. Thus, by directly comparing the kinetics of annealing of each probe when hybridized in parallel to preparations of control and experimental DNA, potentially spurious effects due to significant variations in DNA size distribution will be obviated so that a relative displacement of C_0t curves should reflect an actual concentration difference in the specific sequence under study in the two driver DNA preparations.

DNase I Sensitivity of the Ovomucoid Gene and Its Adjacent Nontranscribed Flanking Sequences. A simplified restriction map of the natural ovomucoid gene and its 5′ and 3′ flanking sequences is shown in Figure 1A. Digestion of the 13.7-kb (*Eco*RI$_a$/*Eco*RI$_b$) restriction fragment with *Hin*dIII generates seven additional fragments. The ones used in the present study are designated OM2.0, OM2.1, OM4.0, OM1.1, and OM3.4 and have been ordered as shown (Lai et al., 1979). (In addition, fragments of 0.8 kb located between OM1.1 and OM3.4 and of 0.3 kb, whose location has not been established, are also produced.) Digestion of the 6.0-kb (*Eco*RI$_b$/*Eco*RI$_c$) fragment with *Bam*HI and *Mbo*II generates two fragments designated OM0.8 and OM5.0 (ordered as shown) and a third fragment of 0.2 kb which was also not utilized here (Lai et al., 1979).

Experiments recently conducted in our laboratory in which these fragments were hybridized to nuclear RNA isolated from estrogen-primed chicks to determine the extent, if any, of their expression in vivo, as well as studies directed toward a precise identification of the 5′ and 3′ ends of the primary transcript using a mapping technique similar to that of Berk & Sharp (1977), have established that the initiation and termination sites for transcription correspond closely, if not exactly, to those sequences coding for the 5′ and 3′ termini of mature ovomucoid messenger RNA (D. Roop, unpublished results). To investigate whether the adjacent 5′ and 3′ nontranscribed regions which immediately flank the ovomucoid gene differ in DNase I sensitivity compared to regions known to be transcribed in vivo, we labeled the restriction fragments described above (Figure 1A) by nick translation and hybridized them either to DNA isolated from nuclei digested in situ with DNase I (henceforth referred to as DNase I digested DNA) or to control DNA prepared as described above.

The kinetics of annealing of the ovomucoid DNA sequences corresponding to OM1.1 and OM3.4, as determined by standard C_0t analysis, are presented in parts A and B of Figure 3, respectively. These sequences, both of which lie entirely within the transcription domain, are preferentially digested in situ compared to the bulk genomic DNA as indicated by an increase in equivalent C_0t values required to achieve comparable levels of probe saturation with DNase I digested driver DNA relative to control DNA. These data have been transformed as shown in parts C and D of Figure 3 by plotting $H/(1 - H)$ against C_0t where H represents the fraction of tracer driven into hybrid (as determined by resistance to S1 nuclease) relative to the maximum hybridization observed. This method of analysis generates linear plots from second-order kinetic data, thereby facilitating quantitative comparisons since the concentration of tracer sequence in a driver preparation is proportional to its slope (Britten et al., 1974; Garel

Table I: DNase I Sensitivity of DNA Sequences Associated with Specific Gene Domains in Hen Oviduct Nuclei

gene	sequence probed[a]	concn of sequence in control DNA relative to DNase I digested DNA[b]
ovomucoid	transcribed DNA	
	OM1.1	2.7
	OM3.4	2.6, 2.1, 2.1
	5′ flanking DNA	
	OM2.0	2.2, 2.7, 2.3
	OM2.1	3.5, 2.6
	OM4.0 (92% flanking sequence)	2.5
	3′ flanking DNA	
	OM0.8	3.0
	OM5.0	2.5
ovalbumin	transcribed DNA	
	OV2.4	2.2, 2.3, 2.5
	OV1.8	2.3, 2.4
	OV2.7 (81% transcribed sequence)	2.3
	5′ flanking DNA	
	OV5.5 (94% flanking sequence)	2.3
	3′ flanking DNA	
	OV3.2	2.1, 2.5
	OV1.7	2.6
Y gene	transcribed DNA	
	Y1.2	2.3
X gene	transcribed DNA	
	X4.5	2.2
	5′ flanking DNA	
	X1.5/1.7	2.3
	X2.2	2.6
β-globin	transcribed and flanking DNA	
	βGL6.1	0.71, 0.65, 0.85

[a] Relative location of specific DNA restriction fragments used as hybridization probes is presented in Figure 1 for the ovomucoid, ovalbumin, X, and Y gene sequences. The βGL6.1 fragment is described by Ginder et al. (1979). [b] Ratios calculated from slopes of C_0t vs. $H/(1-H)$ plots as described in the text. Multiple values for a given sequence represent results obtained from independent experiments.

& Axel, 1976). Data analysis from three independent experiments indicated that the nuclease sensitivity of these transcribed sequences was approximately 2.5-fold greater than that of the bulk DNA (Table I). This observation is in accord with previous studies on the DNase I sensitivity of structural regions of the ovalbumin gene in oviduct nuclei (Garel & Axel, 1976; Palmiter et al., 1977; Bellard et al., 1977). The contention that these sequences are in fact preferentially attacked is further supported by the observation that the chicken β-globin natural gene, which in contrast to the ovomucoid gene is not actively expressed in the oviduct, did not exhibit preferential nucleolytic sensitivity (parts A and B of Figure 4), and in each of three separate experiments it was slightly enriched in the DNase I digested preparation compared to the control (Table I). Since the OM1.1 and OM3.4 fragments are comprised predominantly (ca. 80%) of intervening sequence DNA, it can be concluded that the intervening as well as the structural sequences exist in a DNase I sensitive conformation, a result consistent with the finding that the intervening sequences of the ovomucoid gene are transcribed in vivo (Nordstrom et al., 1979).

Analogous data were obtained by using probes to the 5′ nontranscribed flanking DNA (OM2.0, OM2.1, and OM4.0) and the 3′ nontranscribed flanking DNA (OM0.8 and OM5.0) and are presented in Figure 5 (A–E, respectively). [The

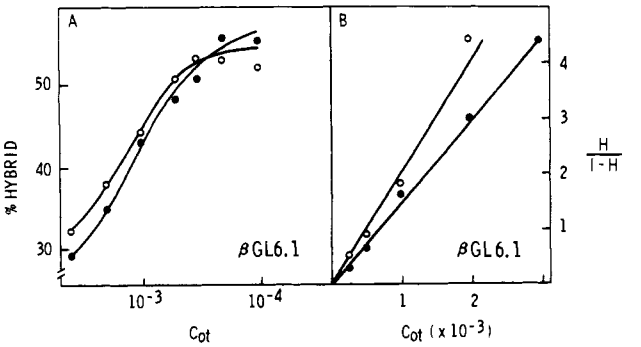

FIGURE 4: DNase I resistance of β-globin genomic DNA sequences in oviduct nuclei. A restriction fragment containing the chicken β-globin natural gene (βGL6.1) was labeled by nick translation and hybridized to preparations of DNase I digested (○) and control DNA (●). Driver DNA was at a concentration of 10 mg/mL. (A) Kinetics of annealing as determined by standard C_0t analysis. (B) Transformed annealing data.

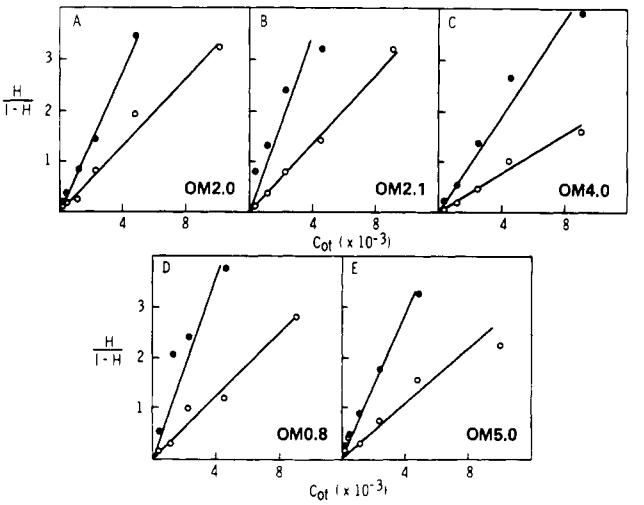

FIGURE 5: DNase I sensitivity of nontranscribed sequences which flank the 5′ and 3′ ends of the ovomucoid gene in oviduct nuclei. Labeled restriction fragments comprising various DNA sequences flanking the ovomucoid gene were hybridized to preparations of DNase I digested (○) and control DNA (●). Driver DNA concentrations were identical in all cases (10 mg/mL). Transformed annealing data are presented. The 5′ flanking fragments were as follows: (A) OM2.0, (B) OM2.1, (C) OM4.0 (OM4.0 is approximately 90% 5′ flanking sequence DNA and 10% transcribed DNA). The 3′ flanking fragments were as follows: (D) OM0.8, (E) OM5.0. The relative location of these restriction fragments is depicted in Figure 1A.

OM4.0 fragment is not entirely a flanking sequence since approximately 0.3 kb of the 3′ terminus is transcribed in vivo (D. Roop, unpublished results); however, for practical purposes its nucleolytic sensitivity will be indicative of nontranscribed flanking DNA.] The relative differences in annealing kinetics observed with these probes are comparable to those obtained with probes from within the transcription domain. These findings, which are summarized in Table I, indicate that all of the ovomucoid regions tested exhibited approximately the same extent of nuclease sensitivity and suggest that those as yet elucidated aspects of chromatin structure which confer DNase I sensitivity to an expressed, or potentially expressible, gene are not necessarily confined within the boundaries of transcription. In the case of the ovomucoid gene this sensitivity extends at least 7.8 kb upstream from the 5′ end of the gene and at least 5.8 kb downstream from the 3′ end. Accounting for the 5.6-kb length of the natural ovomucoid gene (Lai et al., 1979), it appears that at least a 19.2-kb fragment of DNA which includes the ovomucoid gene is entirely in a DNase I

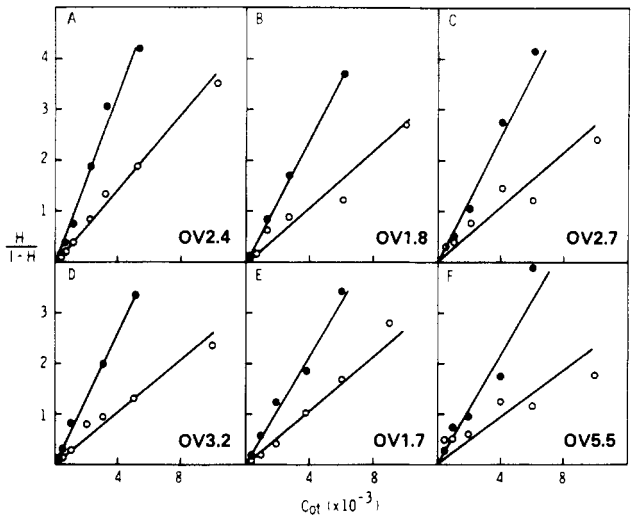

FIGURE 6: DNase I sensitivity of transcribed and nontranscribed 5′ and 3′ flanking sequences of the natural ovalbumin gene in oviduct nuclei. Labeled restriction fragments comprising transcribed regions of the ovalbumin gene as well as various 5′ and 3′ flanking DNA sequences were hybridized to preparations of DNase I digested (○) and control DNA (●). Driver DNA preparations were at concentrations of either 7.5 or 10 mg/mL and were invariant for the hybridizations shown in a given panel. The specific sequences probed were as follows: (A) OV2.4, (B) OV1.8, (C) OV2.7, (D) OV3.2, (E) OV1.7 (two unresolved adjacent fragments), (F) OV5.5. The relative location of these restriction fragments is depicted in Figure 1B.

sensitive conformation in hen oviduct nuclei.

DNase I Sensitivity of the Ovalbumin Gene and Its Adjacent Nontranscribed Flanking Sequences. To determine if DNase I sensitivity of the nontranscribed flanking sequences may be a general property of tissue-specific genes, we conducted studies similar to those described above with probes complementary to various segments included within and flanking the ovalbumin gene. A simplified restriction map of this gene and its flanking DNA is shown in Figure 1B. The OV2.4-kb (*Eco*RI_L/*Eco*RI_M), OV1.8-kb (*Eco*RI_M/*Eco*RI_N), and OV9.2-kb (*Eco*RI_N/*Eco*RI_O) restriction fragments have been described in detail (Dugaiczyk et al., 1979; Gannon et al., 1979). The OV2.4 and OV1.8 fragments are entirely included within the ovalbumin gene whereas OV9.2 includes the terminal 2.2 kb of the 3′ end of the gene and approximately 7 kb of 3′ flanking sequence (Dugaiczyk et al., 1979). Digestion of this fragment by *Hin*dIII generates additional four fragments (designated OV2.7, OV3.2, and two OV1.7) which are ordered as shown (Roop et al., 1980). The two 1.7-kb fragments have not been resolved from one another so that studies conducted with this mixed probe are specific to the 3.4-kb region comprised by the two fragments. Moreover, an OV5.5-kb (*Eco*RI_J/*Eco*RI_K) fragment which contains approximately 5.1 kb of 5′ flanking sequence has been isolated from a chicken DNA library and previously described (Dugaiczyk et al., 1979). These fragments, and others not detailed here, have been utilized in our laboratory to delineate the transcription domain of the ovalbumin gene, and the results using several experimental approaches have consistently indicated that as for ovomucoid, the initiation and termination sites for transcription are coincident with those sequences coding respectively for the 5′ and 3′ termini of the mature ovalbumin messenger RNA (Roop et al., 1980; Tsai et al., 1980).

These restriction fragments were labeled by nick translation and used to probe the relative DNase I sensitivity of the ovalbumin gene and its nontranscribed flanking sequences. As seen from the data presented in parts A and B of Figure 6, the sequences corresponding to OV2.4 and OV1.8, which are both transcribed in their entirety, are preferentially attacked by DNase I as expected; moreover, the extent of preference is similar to that observed above for the ovomucoid gene. The 3′ nontranscribed flanking region comprised of OV3.2 and the two OV1.7 fragments is likewise preferentially digested in situ by DNase I (parts D and E of Figure 6) as is OV2.7 which spans the termination site and thus includes both transcribed and nontranscribed sequences (Figure 6C). Comparable results were obtained with the OV5.5 probe which is comprised of approximately 95% nontranscribed flanking sequences (Figure 6F). The data from three separate experiments are summarized in Table I. By use of a value of 7.6 kb for the size of natural ovalbumin gene (Dugaiczyk et al., 1979), it is concluded that a fragment of DNA at least 19.8 kb in length which contains the ovalbumin gene and approximately 5.2 and 7.1 kb of 5′ and 3′ flanking sequence, respectively, exists in a DNase I sensitive conformation in the oviduct nucleus.

DNase I Sensitivities of the Ovalbumin-Related X and Y Genes. The ovalbumin gene has been shown to exist within a cluster of closely linked genes which includes at least two genes (designated X and Y) which are located 5′ proximal to the ovalbumin gene (Royal et al., 1979) as shown in Figure 1B. These genes share limited homologies with the ovalbumin gene and are apparently expressed in vivo although their potential function remains obscure. They are induced by steroid hormones in vivo (Royal et al., 1979), but the maximal transcription rate for X and Y is only 1 and 6%, respectively, that of ovalbumin (Colbert et al., in press). Clones containing the X and Y genes as well as their flanking sequences have recently been characterized in our laboratory. These include a Y1.2-kb (*Eco*RI_H/*Eco*RI_I) restriction fragment comprising a region of Y which shares no apparent sequence homology to ovalbumin, an X4.6-kb (*Eco*RI_D/*Eco*RI_E) restriction fragment comprising a region of X which shares only limited sequence homology with ovalbumin, an X1.5/1.7-kb probe composed of two unresolved restriction fragments (*Eco*RI_C/*Eco*RI_D and *Eco*RI_F/*Eco*RI_G) composed predominantly of X gene 3′ and 5′ flanking sequence, and an X2.2-kb (*Eco*RI_A/*Eco*RI_B) fragment which is entirely X gene 5′ flanking sequence DNA (Colbert et al., in press). The relative locations of these fragments are shown in Figure 1B. When these fragments were labeled and hybridized to DNase I digested and control DNA prepared from hen oviduct nuclei, the annealing data indicated that these regions also exist in a DNase I sensitive conformation (Figure 7) and that the degree of sensitivity is comparable to that observed for the ovomucoid and ovalbumin specific sequences (Table I).

DNase I Sensitivities of Ovomucoid-, Ovalbumin-, and Globin-Specific Sequences in Nuclei Isolated from Other Tissues. As an additional control for validating the above observations and facilitating interpretation of the data, selected ovomucoid- and ovalbumin-specific probes as well as the β-globin probe were hybridized to DNase I digested and control DNA prepared from spleen, liver, and erythrocyte nuclei in a manner analogous to that described above for oviduct nuclei. The electrophoretic profiles of the DNA preparations utilized in these studies are shown in Figure 2 (lanes 3–8) and demonstrate that the size distributions of the DNase I digested and control DNA preparations from each tissue were roughly comparable. In spleen nuclei, sequences comprising the ovomucoid gene as well as sequences flanking the 5′ and 3′ ends of this gene were resistant to DNase I (parts A–D of Figure 8). Similar results were obtained with ovalbumin- and globin-specific probes (parts E and F of Figure 8). Likewise,

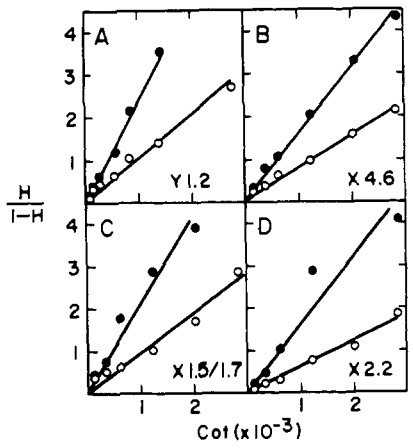

FIGURE 7: DNase I sensitivity of the ovalbumin-related X and Y genes in oviduct nuclei including X gene and 5′ flanking sequences. Labeled restriction fragments comprising transcribed regions of the Y gene (Y1.2; panel A) and the X gene (X4.6; panel B) which share, respectively, no apparent sequence homology or limited sequence homology with the ovalbumin gene and fragments comprised predominantly (X1.5/1.7; panel C) or entirely (X2.2; panel D) of X gene 5′ flanking DNA were hybridized to preparations of DNase I digested (O) or control DNA (●) which were at concentrations of 8 mg/mL in all cases. The relative location of the restriction fragments is depicted in Figure 1B.

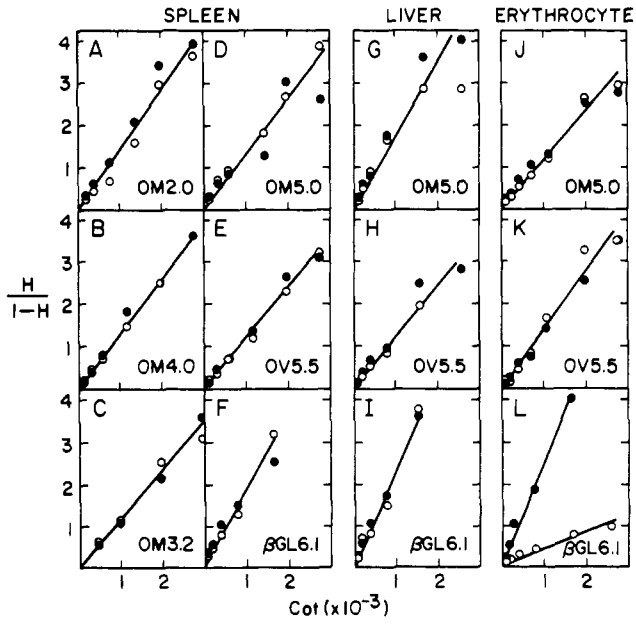

FIGURE 8: DNase I sensitivity of ovomucoid-, ovalbumin-, and globin-specific sequences in control nuclei isolated from other tissues. DNase I digested (O) and control DNA (●) prepared from nuclei of chick spleen (A–F), chick liver (G–I), and hen erythrocyte (J–L) was hybridized to the appropriate sequence probes as follows: (A) OM2.0, (B) OM4.0, (C) OM3.2, (D) OM5.0, (E) OV5.5, (F) βGL6.1, (G) OM5.0, (H) OV5.5, (I) βGL6.1, (J) OM5.0, (K) OV5.5, (L) βGL6.1. Driver DNA concentrations were 8 mg/mL in all cases.

ovomucoid, ovalbumin, and globin sequences were resistant to DNase I in liver nuclei (parts G–I of Figure 8). In erythrocyte nuclei, ovomucoid and ovalbumin sequences were resistant to DNase I (parts J and K of Figure 8), whereas the globin sequence, in contrast, was DNase I sensitive (Figure 8L), an observation in accord with previous findings (Weintraub & Groudine, 1976). These data are consistent with the concept that the specific gene domains under study are DNase I insensitive in those tissues in which their constituent genes are not, or have never been, expressed and further suggest that the observed DNase I sensitivities of the ovomucoid and ovalbumin domains are related to the expression of these tissue-specific genes in the oviduct.

Discussion

Pancreatic DNase I has previously been shown to preferentially digest a variety of transcriptionally active genes in chromatin. These include globin (Weintraub & Groudine, 1976), ovalbumin (Garel & Axel, 1976; Bellard et al., 1977; Palmiter et al., 1977; Bloom & Anderson, 1979), *Drosophila* heat shock genes (Biessmann et al., 1977; Wu et al., 1979), ribosomal genes (Mathis & Gorovsky, 1977; Stadler et al., 1978), and a diversity of integrated viral genes (Panet & Cedar, 1977; Flint & Weintraub, 1977; Groudine et al., 1978). Transcriptional activity per se is apparently not required to maintain the "active" conformation since genes which were once expressed but subsequently inactive during normal development or in response to (or withdrawal from) acute environmental stimuli can still manifest the sensitive conformation (Weintraub & Groudine, 1976; Palmiter et al., 1977; Beissmann et al., 1977; Miller et al., 1978). Moreover, a gene's degree of sensitivity to DNase I appears to be independent of the rate at which it is transcribed (Garel et al., 1977). These observations are consistent with the concept that sensitivity to DNase I may be a necessary but not a sufficient condition for transcription.

Since the probes used in most of these studies were specific only to structural regions of the gene known to be transcribed in vivo, regulation of transcriptional activity does not seem to involve an acute transition between DNase I sensitive and insensitive states which propagates throughout the entire gene. However, little information is available concerning the conformation of the DNA which directly flanks a gene. Since the flanking DNA is presumably never transcribed in vivo, it seemed conceivable that these sequences might exist perpetually in a DNase I insensitive conformation and that discrete transitions in chromatin conformation occurring at the 5′ and 3′ ends of the gene might play a physiological role in delineating the boundaries of the transcription unit. Evidence for such a mechanism has been reported by Flint & Weintraub (1977), who detected a transition from active to inactive chromatin which occurred within a few nucleosomes at the transcription boundary of an integrated adenovirus gene actively expressed in transformed cells.

Our studies failed to detect such structural transitions at the boundaries of the oviduct-specific genes, ovomucoid and ovalbumin. Continuous lengths of DNA of approximately 19.6 and 19.8 kb containing respectively the ovomucoid and ovalbumin natural genes and their 5′ and 3′ nontranscribed flanking sequences were probed in discrete segments and found to exist entirely in a DNase I sensitive conformation in the hen oviduct nuclei. Since the fragments included within the transcription domains of these two genes consist predominantly of intervening sequence DNA, it can be concluded that the intervening sequences exist in a DNase I sensitive conformation similar to that previously reported for the structural sequences.

The concept that a DNA domain which is never expressed can still exist in a DNase I sensitive conformation is dependent upon the claim that the sequence in question really is transcriptionally inactive. This is particularly true since even genes transcribed at low levels have been shown to exhibit DNase I sensitivity (Garel et al., 1977). Studies in our laboratory strongly suggest that, for both the ovomucoid (D. Roop, unpublished results) and ovalbumin genes (Roop et al., 1980; Tsai et al., 1980), the boundaries of the transcription unit coincide with those sequences which code for the 5′ and 3′ termini of the mature messenger RNA. If transcription of the flanking

DNA does occur, it must be at an extremely low rate since the techniques utilized were capable of detecting a steady-state level of much less than 0.1 RNA transcript/tubular gland cell nucleus. Alternatively, it might be argued that flanking sequence DNA is actually transcribed at a significant level but is subsequently processed away so rapidly that its steady-state concentration fails below the threshold of detection. This possibility seems unlikely in view of the fact that spectra of processing intermediates have been detected from transcripts of the ovomucoid (Nordstrom et al., 1979) and ovalbumin genes (Roop et al., 1978) and have been shown to contain various intervening sequences. The failure to detect sequences complementary to flanking sequence DNA in any of these precursors would require that, if originally included in the primary transcript, their rate of excision and degradation far exceed that of the intervening sequences. It seems more reasonable to postulate that if transcription of the DNA flanking these two genes occurs at a level which cannot be detected with the available methodology, then that level of expression probably has no physiological function. If so, then the results presented here suggest the existence in chromatin of DNA sequences which in all likelihood are never destined to be transcribed and yet which are organized into a DNase I sensitive conformation.

It should be noted that although some progress has been made toward elucidating the molecular basis of DNase I sensitivity (Weisbrod & Weintraub, 1979; Weisbrod et al., 1980), the designation of a chromatin domain as either DNase I sensitive or resistant still remains an operational one. Thus, failure to detect a difference in DNase I sensitivity between a transcribed gene and its nontranscribed flanking DNA might indicate that under the conditions utilized structural subleties delineating the transcription unit from its flanking sequences may have escaped detection. The existence of such structural differences is in fact suggested by recent reports that micrococcal nuclease, which exhibits a different cutting specificity in chromatin, discriminates between transcriptionally active and inactive states (Bloom & Anderson, 1979; Johnson et al., 1979). Moreover, extremely limited digestions with DNase I have been employed to detect hypersensitive cleavage sites in gene domains which may be related to perturbations in higher order chromatin organization in the neighborhood of the gene (Wu et al., 1979; Kuo et al., 1979). Since these latter studies employed between 1 and 2 orders of magnitude less digestion than those reported here, it is not evident at present what relation, if any, these hypersensitive sites bear to the sensitive regions observed following more extensive digestion. However, it can be concluded from two lines of evidence presented herein that the nontranscribed sequences flanking these active tissue-specific genes do exist in an altered conformation rendering them susceptible to preferential attack by DNase I. (1) Preferential sensitivity was not observed for other domains containing a nonexpressible gene in the same nucleus. (2) Preferential sensitivity of the flanking sequences was observed only in the tissue in which their enclosed genes are expressed. It seems reasonable to postulate that the DNase I sensitivity of the flanking sequences is related to their spacial proximity to transcribed genes and may play some role in the transcription process.

From the available data it is not possible to estimate the percentage of DNA which might be included in the DNase I sensitive category. Within the limited DNA domains investigated here, nontranscribed sequences exceeded those that are transcribed by at least twofold. However, this value is subject to considerable uncertainty and may depend on the frequency of occurrence and relative spacing of multiple genes in the same neighborhood. Furthermore, the ovalbumin and ovomucoid genes are tissue specific and the conformation of their flanking sequences may not be typical of genes serving less specialized functions. Even so, it seems likely that the fraction of DNA sensitive to DNase I might significantly exceed that fraction which is actually transcribed. Since it has also been reported that about 18% of the duplex DNA is transcribed into nuclear RNA in the hormonally stimulated oviduct (Liarakos et al., 1973), the amount of DNA organized in the DNase I sensitive conformation may comprise a significant fraction of the genome. It is interesting in this respect that in yeast, whose genome is expressed proportionately at an unusually high level compared to more advanced eukaryotes, the entire genome has been reported sensitive to DNase I (Lohr & Hereford, 1979).

Finally, the availability of extended DNA fragments containing multiple unique genes should provide further insight into the relationship between gene structure and expression. The estrogen-induced X and Y genes are located approximately 23 and 11 kb 5′ proximal to the ovalbumin gene (Royal et al., 1979), but their maximal levels of expression are only 1 and 6%, respectively, that of ovalbumin (Colbert et al., in press). The DNase I sensitivity of the X and Y genes is comparable to that observed for the ovalbumin gene, a result in agreement with Garel et al. (1977), who used a mixed probe prepared against oviduct poly(A) RNA sequences present in low frequence. Reference to Figure 1B indicates that within the 54 kb of DNA bounded by restriction sites *Eco*RI$_A$ and *Eco*RI$_O$, 30 kb has been shown to exist in a DNase I sensitive conformation in oviduct nuclei. Since the sequences probed comprise representative transcribed and nontranscribed regions, it seems likely that the entire 54 kb is DNase I sensitive. If it is assumed that the transcribed as well as the nontranscribed DNA is organized into nucleosomes (Foe et al., 1976; Kuo et al., 1976; Reeves, 1976) which have a mean periodicity approximating that observed in most eukaryotic tissues (i.e., 200 base pairs; Kornberg, 1977), then our results would suggest that ovalbumin and its related genes are included within a strand of at least 270 nucleosomes, all of which apparently exist in a DNase I sensitive conformation. These values represent minimum estimates for the actual situation in vivo since probes for more distal regions of the ovalbumin domain are not presently available.

Although the total length of DNA continuously packaged in an active conformation remains to be determined, these data suggest the possibility that polygenic domains may be recruited through a deterministic or differentiative process into a DNase I sensitive state which encompasses the entire domain including the nontranscribed intergenic spacers. Within this DNase I sensitive and potentially expressible domain, effectors could then operate on this primed network to modulate the expression of individual genes.

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