The association of transcriptionally active genes with the nuclear matrix of the chicken oviduct

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ABSTRACT

Eucaryotic DNA is organized into a series of supercoiled loops that are anchored to the nuclear matrix. When these DNA loops are cleaved by endonucleases, the DNA sequences which remain associated with the nuclear matrix can be recovered and analyzed for their content of specific genes. Using restriction endonucleases to cleave the loops, we demonstrate that ovalbumin and conalbumin gene sequences are preferentially associated with the nuclear matrix of hen oviduct cells but not with the nuclear matrix of hen brain cells. Furthermore, we determined that several regions of the ovalbumin gene were independently attached to the nuclear matrix of hen oviduct cells. This included sequences located 3.8 kb downstream from the 3' end of the ovalbumin gene transcription unit. To determine whether the nuclear matrix association of the ovalbumin gene was regulated by hormones, we examined the oviduct cells of chicks that underwent primary estrogen stimulation, estrogen withdrawal and secondary estrogen stimulation. Ovalbumin gene sequences selectively dissociated from the chick oviduct nuclear matrix during estrogen withdrawal and reassociated with the nuclear matrix following restimulation.

INTRODUCTION

Several levels of DNA organization have been discovered in the interphase nucleus. At one level, the DNA is arranged into supercoiled loops¹⁻³ that are anchored to sites on the nuclear matrix⁴. The nuclear matrix is a residual framework structure that has been identified in a variety of eucaryotic cell types (reviewed in reference 5). DNA loops have been directly visualized, using fluorescence or electron microscopy, as a halo of DNA which surrounds the central nuclear matrix core^{4,6,7}.

Previous studies have shown that certain DNA sequences are nonrandomly arranged within supercoiled DNA loops with respect to the nuclear matrix anchorage sites (reviewed in reference 8). In particular it has been shown that transcriptionally active genes are arranged nonrandomly and appear closely associated with the nuclear matrix⁹⁻¹³. These observations suggested that the nuclear matrix might be playing a role in the regulation or mechanics of gene transcription. In the present study several questions relating

to this role were addressed. First, selected genes were systematically examined in the oviduct and brain cells of egg-laying hens, using restriction endonucleases to cleave the halo of DNA loops. The results indicated that all transcribed genes examined were apparently associated with the nuclear matrix while nontranscribed genes were not. Furthermore, this association was independent of the nuclease used to prepare the structures and was not due to resistance of transcribed sequences to cleavage by such nucleases. Next, the ovalbumin gene was examined at a more detailed level in hen oviduct cells to determine where the matrix attachment sites were located. This analysis indicated that the association of the ovalbumin gene with the nuclear matrix was mediated by nuclear matrix attachment sites in several regions of the ovalbumin gene and in a region located approximately 3.8 kb downstream from the 3' end of the gene. Finally, the transcriptionally regulated ovalbumin and conalbumin genes were examined in the oviduct cells of chicks that had undergone primary estrogen stimulation, acute estrogen withdrawal and secondary estrogen stimulation. These studies showed that the nuclear matrix association of the ovalbumin gene in chick oviduct cells was dependent on the chicks' hormonal status.

MATERIALS AND METHODS

Animals

Egg-laying White Leghorn hens or female chicks were used (Truslow Farms, Chestertown, Maryland). For primary estrogen stimulation, 14 day old chicks were subcutaneously implanted with two 15 mg diethylstilbestrol (DES) pellets for 14 days. For acute estrogen withdrawal, the pellets were removed 14 days after implantation and the chicks sacrificed 48 hours later. For secondary estrogen stimulation, chicks were injected intramuscularly with 5.0 mg DES dissolved in 0.4 ml sesame oil 48 hours after pellet removal. Chicks were stimulated for 10 hours. Six or seven chick oviducts were pooled for each matrix preparation.

Preparation of Nuclei and Nuclear Matrix

After decapitation, the magnum section of the oviduct or entire brain was excised. Nuclei were partially purified by Dounce homogenization in IS buffer (100 mM NaCl, 50 mM KCl, 20 mM Tris-HCl, 5 mM MgCl₂, pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride and 0.2% methylmethanethiosulfonate [as sulfhydryl blocker and protease inhibitor¹⁴]) containing 0.25 M sucrose and 1.0% Nonidet-P40. Following filtration through a nylon screen, the homogenate was pelleted at 330 x g through a cushion of IS buffer containing 0.5 M sucrose.

Nuclei were resuspended in IS buffer and an equal volume of HS buffer (4.0 M NaCl, 20 mM Tris-HCl, 5 mM MgCl₂, pH 7.4) was added to give a final nuclear matrix concentration of $1.0 - 1.5 \times 10^6$ /ml. As described previously, the nuclei appear as "matrix-halo" structures after this treatment⁴.

Matrix-halo structures were loaded onto step gradients containing 60% and 4% glycerol layers in NM buffer (2.0 M NaCl, 20 mM Tris-HCl, 5 mM MgCl₂, pH 7.4). After centrifugation at 4000 x g for 1 to 2 hours, the structures were recovered from the interface separating the 60% and 4% glycerol layers. For EcoRI cleavage, the matrices were resuspended in 40 volumes of 50 mM Tris-HCl, 10 mM MgCl₂, pH 7.5 to adjust the ionic strength to 50 mM NaCl. For XbaI cleavage the matrices were resuspended in 20 volumes of 6 mM Tris-HCl, 6 mM MgCl₂, pH 7.5 to adjust the ionic strength to 100 mM NaCl. EcoRI or XbaI (Bethesda Research Laboratories, Rockville, Maryland) was added to matrix suspensions at a concentration of 20 units/ml. Digestion proceeded at 37°C for 1 hour. Matrices were recovered by centrifugation at 9000 x g for 20 minutes. Unless otherwise stated, all steps were performed at 4°C. Morphology was followed throughout the procedure using phase and fluorescence microscopy⁴.

Analysis of DNA and RNA

DNA from whole nuclei, nuclear matrices or supernatant solutions was purified by the method of Gross-Bellard *et al.*¹⁵. DNA concentrations were determined by a fluorometric assay¹⁶. Unless otherwise stated, purified DNA samples were cleaved with a 5-fold excess of EcoRI or XbaI for 16 hours at 37°C in the manufacturer's recommended buffering solution. Agarose gel electrophoresis, transfer to nitrocellulose, hybridizations¹⁷, and densitometric quantitation of autoradiograms were performed as previously described¹³. [³²P]-labeled hybridization probes with specific activities of $0.5 - 3 \times 10^9$ dpm/ug DNA were prepared by nick translation¹⁸ or the "oligolabeling" technique¹⁹. In some instances filters were prepared for rehybridization to additional probes by the method of Parada *et al.*²⁰. Probes were prepared from the following recombinant molecules or their excised inserts: pOvE12 (an ovalbumin cDNA clone containing sequences of internal exons but not the leader exon)²¹, pHb1001 (adult beta-globin cDNA)²²; pCon (conalbumin cDNA)²³; p4/322 (apo-VLDL II genomic fragment)²⁴; pOv (ovalbumin cDNA)²⁵. pOv-1,4,6 and 7 (see Fig. 5) were supplied by P. Chambon: pOv-1 was a Pst I/AvaII fragment from the 5' flanking region of the gene; pOv-4 (a HinfI/HindIII fragment), pOv-6 (a HindIII/BamHI fragment) and pOv-7 (a HindIII/EcoRI fragment) were both from the 3' flanking region of the gene.

Ovalbumin and conalbumin mRNA levels were determined by Rot analysis as previously described²⁶. In vitro transcription of isolated nuclei was used to directly measure the transcriptional rate of the ovalbumin and conalbumin genes²⁷. Transcription rates are expressed as the fraction of total RNA synthesis specific for a given cloned sequence. Results were corrected for background hybridization to pBR322 (generally less than 0.001% of input) and efficiency of filter hybridization (15-30%) as measured by internal [³H] cRNA standards. Results are given in parts per million (ppm).

RESULTS

Active, but not inactive, genes are enriched in hen oviduct nuclear matrices.

Sequences associated with the nuclear matrix were isolated and analyzed in the following way. Detergent treated nuclei were extracted with 2M NaCl to remove histones and most other nuclear proteins. The supercoiled DNA loops that are anchored to the resulting nuclear matrix structures were then cleaved with the restriction endonuclease EcoRI. After centrifugation, detached DNA sequences remained in the supernatant while matrix-associated DNA sequences ("matrix DNA") were recovered from the pellet. Once purified, matrix DNA was assessed for its content of specific gene sequences by a quantitative application of the Southern blotting technique. Samples were redigested with EcoRI, electrophoresed through agarose gels, transferred to nitrocellulose filters, hybridized to [³²P]-labeled probes and examined by autoradiography. To quantitate the relative abundance of a specific gene sequence in matrix preparations, reference samples of EcoRI cleaved total nuclear DNA were included on each filter. As shown previously, the intensity of the autoradiographic band obtained for a given fragment is linearly related to its sequence content over at least an 8-fold range.¹⁰

In previous studies from our laboratory, the supercoiled loops which are anchored to the nuclear matrix were cleaved with DNase I^{8,10,13}. The percentage of total nuclear DNA contained in each matrix preparation was dependent on the extent of DNase I treatment. To obtain reproducible matrix preparations (i.e., preparations containing similar percentages of nuclear DNA after DNase I treatment), the progress of the DNase I reaction had to be closely monitored. When we used EcoRI instead of DNase I to cleave the matrix-attached DNA loops, similar end points of digestion were consistently achieved. Typically, 8 +/- 2% of the nuclear DNA was recovered from chicken brain or oviduct nuclear matrices that were prepared with EcoRI.

A reliable comparison could therefore be made between the hen oviduct

Figure 1

Expressed and non-expressed gene sequences in nuclear matrix-associated DNA.

Matrix-halo structures from hen oviduct or brain cells were cleaved with EcoRI and the DNA from pelleted matrices was isolated, purified and redigested with EcoRI as described in Materials and Methods. 1.5 ug of hen oviduct (lane Ov) or brain (lane Br) matrix DNA and various amounts of EcoRI cleaved total nuclear DNA (0.5 = 0.75 ug; 1 = 1.5 ug; 2 = 3.0 ug; 4 = 6.0 ug) were electrophoresed through 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized to the following probes:

- (A) ovalbumin cDNA insert from pOvE12
- (B) conalbumin cDNA insert from pCon
- (C) beta-globin cDNA insert from pHb1001
- (D) p4/322 containing a genomic apo VLDL II insert

matrix preparation and the hen brain matrix preparation that were analyzed in Figure 1. Several identical filters were prepared, each containing 1.5 ug of oviduct matrix DNA, 1.5 ug of brain matrix DNA and 0.75 ug, 1.5 ug, 3.0 ug and 6.0 ug of EcoRI cleaved oviduct nuclear DNA. The filters were independently hybridized to several probes, some detecting genes which are transcribed in the oviduct but not in the brain, others detecting genes which are not transcribed in either tissue. Autoradiograms of filters that were hy-

bridized to probes which detect the ovalbumin and conalbumin genes (genes which are transcribed in the oviduct but not in the brain) show that hen oviduct matrix DNA contained a greater content of these genes than an equivalent amount of hen brain matrix DNA or total nuclear DNA (Fig. 1A and B). In fact, 1.5 ug of oviduct matrix DNA contained as much of the ovalbumin and conalbumin genes as 4.5-8.0 ug of total nuclear DNA (range of six experiments). In contrast, 1.5 ug of brain matrix DNA contained approximately the same amount of these genes as 0.5-1.25 ug of total nuclear DNA. Similar results (not shown) were obtained with filters that were hybridized to a probe which detects the ovomucoid gene (another gene which is transcribed in the oviduct but not in the brain).

Autoradiograms of filters that were hybridized to probes which detect the beta-globin and apo VLDL II genes (genes which are not transcribed in the oviduct or brain) show that both hen oviduct matrix DNA and hen brain matrix DNA contained a lower content of these genes than an equivalent amount of total nuclear DNA (Fig. 1C and D). In fact, 1.5 ug of oviduct and brain matrix DNA typically contained as much of these two genes as 0.5-1.25 ug of total nuclear DNA.

These results, which were consistent with the results of the studies using DNase I¹³, showed that genes were enriched in nuclear matrix DNA from cells in which they were transcribed, but were not enriched in nuclear matrix DNA from cells in which these genes were inactive. The inactive genes served as technical controls in these experiments. In particular, the lack of enrichment of the beta-globin gene fragment of 9.6 kb and the VLDL gene fragment of 10 kb provided excellent comparisons for the enriched ovalbumin and conalbumin gene fragments of 9.5 and 11.0 kb, respectively.

If there were an enrichment of a specific gene sequence in matrix-associated DNA, then there should be a corresponding depletion of that sequence in the DNA that was detached from the nuclear matrix during EcoRI cleavage. Detached sequences ("supernatant DNA") were recovered from the supernatant after centrifugation of the nuclear matrices. A filter containing 2.0 ug of supernatant DNA redigested with EcoRI after DNA purification (lane S), 2.0 ug of pelleted DNA redigested with EcoRI after DNA purification (lane M), 2.0 ug of pelleted DNA not redigested with EcoRI after DNA purification (lane M*), and 1.0 ug, 2.0 ug and 6.0 ug of EcoRI cleaved total nuclear DNA, was hybridized to a probe which detects the ovalbumin gene. The autoradiogram of this filter shows that while 2.0 ug of the EcoRI redigested pelleted DNA sample contained a substantially higher content of the ovalbumin

Figure 2

The DNA associated with or detached from the nuclear matrix.

Matrix-halo structures from oviduct cells were cleaved with EcoRI. Following centrifugation, the DNA associated with or detached from the nuclear matrix was isolated from the pelleted and supernatant fractions, respectively. After purification, the supernatant DNA and a portion of the pelleted DNA was redigested to completion with EcoRI. 2.0 ug of supernatant DNA (lane S), 2.0 ug of pelleted matrix DNA, either redigested (lane M) or not redigested (lane M*) with EcoRI after purification, were electrophoresed through 0.8% agarose gels with various amounts (0.5 = 1.0 ug; 1 = 2.0 ug; 3 = 6.0 ug) of EcoRI cleaved total nuclear DNA. The DNA was transferred to a nitrocellulose filter and an ovalbumin cDNA insert from pOvE12 was used as a hybridization probe.

gene fragments than 6.0 ug of total nuclear DNA, 2.0 ug of the supernatant DNA sample contained approximately the amount found in 1.0 ug of total nuclear DNA (Fig. 2). When a similar filter was hybridized to a probe which detected the beta-globin gene, the supernatant DNA was neither enriched nor depleted for this gene (not shown). Since the matrix DNA sample used in Figure 2 contained 8% of the nuclear DNA and was enriched approximately 5-fold for the ovalbumin gene, the supernatant DNA should have contained 60% $[100\% - (5 \times 8\%)]$ of the ovalbumin sequence content of total nuclear DNA. This was consistent with the result shown in Figure 2. The reasons that 100% of the ovalbumin genes were not associated with the nuclear matrix may be related to the fact that not all oviduct cells are tubular gland cells producing ovalbumin, as well as other factors described in the Discussion.

Also demonstrated in the autoradiogram of the filter depicted in Fig. 2 was the fact that the digestion of the matrix-halo structures with EcoRI was quite efficient. All three limit digest fragments of the ovalbumin gene (9.5, 2.4, and 1.8 kb [see restriction map of Fig. 5]) were present in the

Figure 3

Ovalbumin gene fragments in hen oviduct nuclear matrix-associated DNA following XbaI cleavage.

Matrix-halo structures from hen oviduct cells were cleaved with XbaI and the DNA from pelleted matrices was isolated and purified. A portion of this purified DNA was subsequently digested to completion with XbaI. 2.0 ug of matrix DNA, either redigested (lane M) or not redigested (lane M*) with XbaI after DNA purification, were electrophoresed through 0.8% agarose gels with various amounts (0.5 = 1.0 ug; 1 = 2.0 ug; 2 = 4.0 ug; 4 = 8.0 ug) of XbaI cleaved total nuclear DNA. The DNA was transferred to nitrocellulose filters and hybridized to the following probes:

(A) pOv-1, containing a genomic insert from the 5' flanking region of the ovalbumin gene.

(B) ovalbumin cDNA insert from pOvE12

(C) pOv-7, containing a genomic insert from the 3' flanking region of the ovalbumin gene

(D) p4/322, containing a genomic apo VLDL II insert

After hybridization to pOv-1 (A) and pOv-7 (C), the probes were removed from the filters and the filters were rehybridized to pOvE12 (B) and p4/322 (D), respectively.

matrix-associated DNA after cleavage of the structures with EcoRI (lane M*). Although the digestion of the matrix-halo structures was efficient, it was not complete, since (i) partially digested fragments in lane M* could be visualized after longer autoradiographic exposure times, and (ii) redigestion

with EcoRI after isolation and purification of the nuclear matrix DNA resulted in a higher enrichment of the limit EcoRI fragments (compare lane M to lane M*), reflecting the conversion of all partially digested fragments to the limit fragment size. The ability to achieve an efficient restriction endonuclease digestion of matrix-halo structures in situ was crucial to the analysis presented below.

Several ovalbumin gene fragments are independently bound to the nuclear matrix

To further analyze the matrix association of the ovalbumin gene, nuclear matrices were isolated from hen oviduct cells using the restriction endonuclease XbaI instead of EcoRI. XbaI was chosen because it cleaves the ovalbumin gene region into several similarly sized fragments, some of which contain transcribed sequences and some of which do not. (See Fig. 5 for the XbaI restriction map of the ovalbumin gene). Filters containing 2.0 ug of matrix DNA (redigested with XbaI [lane M] or not redigested [lane M*] after DNA purification) and 1.0 ug, 2.0 ug, 4.0 ug and 8.0 ug of XbaI cleaved total nuclear DNA were hybridized to several probes which detect different regions within and surrounding the ovalbumin gene. The nuclear matrix preparation analyzed in Figure 3 contained 13% of the nuclear DNA. Autoradiograms of these filters showed that the XbaI redigested oviduct matrix DNA sample (lane M) was enriched relative to an equivalent amount of total nuclear DNA for the 2.4, 2.7, and 4.3 kb fragments which were detected by an ovalbumin cDNA probe (Fig. 3B). These fragments contain central and 3' regions of the ovalbumin gene and 3.8 kb of downstream flanking sequences (Fig. 5). The hen oviduct matrix-associated DNA sample (lane M) was similarly enriched for a 3.5 kb fragment which contained the 5' end of the ovalbumin gene and approximately 2.3 kb of upstream flanking sequences (Fig. 3A). In addition, the oviduct matrix DNA was enriched for a 4.9 kb fragment which contained sequences whose 5' border was located approximately 3.8 kb downstream from the 3' end of the ovalbumin gene (Fig. 3C). When the same filter used in Figure 3C was rehybridized to a genomic probe which detected the nontranscribed apo VLDL II gene, the matrix DNA sample (lane M) was not enriched for sequences containing this gene (Fig. 3D).

Unlike the other fragments that were enriched in matrix DNA, the fragment detected in Figure 3C was outside of the ovalbumin gene transcription unit (see Fig. 5). This was assessed directly by measuring the transcription rate of this region in hen oviduct nuclei. As expected, the ovalbumin gene itself was transcribed at a high rate in hen oviduct nuclei (Table I). The

Table I. Transcription of Genomic Fragments Surrounding the 3' End of the Ovalbumin Gene

	Transcription rate (ppm)	Transcription rate normalized to fragment length of cloned DNA (ppm/kbp)
pOv	2694	1441
pOv-4	485	1212
pOv-6	94	45
pOv-7	35	21

RNA synthesized in isolated hen oviduct nuclei was prepared as described in Materials and Methods and used as a hybridization probe to filters containing the cloned DNA's shown in the Table.

transcription appeared to continue at the same rate beyond the polyadenylation site, and terminated within a region approximately 500 bp distal to this site. This was indicated by the fact that the transcription rate of DNA sequences within pOv-4, a genomic clone of 400 bp whose 5' end is just distal to the polyadenylation site, was indistinguishable from that of the coding region of the ovalbumin gene. Under the incubation conditions used, we estimated that the elongation rate of RNA polymerase was less than 10 nucleotides per minute. Therefore, during our 45 minute incubation of nuclei, polymerases might have artifactually elongated a segment as large as 450 bases past the in vivo termination site. However, when the incubation was limited to 5 minutes, the same relative transcription rate of pOv, pOv-4, and pOv-6 was observed (R. Palmiter, unpublished data), indicating that the polymerase molecules were transcribing past the polyadenylation site in vivo. This observation is consistent with those made on the transcription pattern of other genes²⁸. The abrupt termination of ovalbumin gene transcription in this region was indicated by the striking decrease in labeled nuclear RNA homologous to DNA sequences downstream from this point (see Fig. 5 and Table I).

There was a possible trivial explanation for the enrichment of the 4.9 kb fragment seen in Figure 3C, lane M. Since this fragment is contiguous with the 4.3 kb fragment actually containing the transcribed 3' end of the ovalbumin gene, incomplete digestion of the matrix-halo structures could have produced a 9.2 kb partial digestion fragment which would have contained both the 4.9 and 4.3 kb fragments. These fragments would have been attached to the matrix only by virtue of transcribed sequences lying within the 4.3 kb fragment. Subsequent redigestion of the purified DNA with XbaI would have resulted in the apparent enrichment of the 4.9 kb fragment, even though it

Table II. Transcription of the Ovalbumin and Conalbumin Genes in Hormonally Modulated Chick Oviduct Cells

Hormonal Status of Chicks	OVALBUMIN		CONALBUMIN	
	mRNA (molecules/cell)	Transcription Rate (ppm)	mRNA (molecules/cell)	Transcription Rate (ppm)
Primary Estrogen Stimulation	18,560	1,697	2,100	849
Acute Estrogen Withdrawal	56	25	90	141
Secondary Estrogen Stimulation	2,090	968	790	821

Plasmids used to measure transcriptional parameters were pOv and pCon for ovalbumin and conalbumin, respectively.

was not independently bound to the matrix. To rule out this potential explanation, we examined nuclear matrix DNA after XbaI digestion of the matrix-halo structures without subsequent redigestion of the purified DNA. As shown in Figure 3C, lane M*, the 4.9 kb fragment itself, and not a partially digested larger fragment containing it, was actually enriched in matrix DNA. Comparisons of Figures 3A,B and C show that all fragments analyzed in this region were independently enriched (1.5- to 3-fold) in the matrix DNA obtained directly after XbaI digestion of the matrix-halo structures. Redigestion of the DNA with XbaI after its purification resulted in the complete cleavage of all fragments to the limit size, and increased the enrichment levels of all the fragments to 3- to 4-fold. Hence, matrix-halo structures were digested efficiently (but not completely) with XbaI, and analysis of the results showed that several regions within and surrounding the ovalbumin gene were independently anchored to the nuclear matrix.

Effects of hormonal withdrawal on the association of the ovalbumin gene with the nuclear matrix

The results described above indicated that the ovalbumin gene and other egg white protein genes were anchored to the nuclear matrix in hen oviducts. We wished to determine whether this anchorage was irreversible, i.e., whether it was a permanent feature of the nuclear organization of tubular gland cells, perhaps established during differentiation of the cells and subsequently unresponsive to the physiological status of these cells. To answer this question, we examined nuclear matrices from prematurely stimulated chick oviducts before and after acute hormonal withdrawal.

To achieve hormonal stimulation and withdrawal, we used a protocol similar to that described by other investigators²⁹. The effects of this protocol on gene transcription are listed in Table 2. After two weeks of estrogen

Figure 4

Effect of hormonal stimulation and withdrawal on the matrix association of the ovalbumin gene.

Matrix-halo structures from the oviduct cells of chicks undergoing primary estrogen stimulation, acute estrogen withdrawal or secondary estrogen stimulation were cleaved with EcoRI. The DNA from the pelleted matrices was isolated, purified and redigested to completion with EcoRI. 2.0 ug of DNA from the stimulated (lane S), withdrawn (lane W) or restimulated (lane R) oviduct matrix preparations were electrophoresed through 0.8% agarose gels with various amounts (0.5 = 1.0 ug; 1 = 2.0 ug; 2 = 4.0 ug; 3 = 6.0 ug) of EcoRI cleaved total nuclear DNA. The DNA was transferred to a nitrocellulose filter and was sequentially hybridized to the following probes:

(A) pOv-1, containing a genomic insert from the 5' flanking region of the ovalbumin gene.

(B) ovalbumin cDNA insert from pOvE12

(C) beta-globin cDNA insert from pHb1001

(D) conalbumin cDNA insert from pCon.

stimulation, chick oviduct cells transcribed the ovalbumin gene at a high rate and there was a large cellular ovalbumin mRNA content. After 48 hours of hormonal withdrawal there was a dramatic decrease both in the rate of ovalbumin gene transcription and in ovalbumin mRNA content. Following 10 hours of secondary estrogen stimulation (before any increase in tubular gland cell number²⁹), there was a large increase in the rate of ovalbumin gene transcription and a corresponding increase in ovalbumin mRNA content.

To determine the effects of primary estrogen stimulation, estrogen withdrawal and secondary estrogen stimulation on the nuclear matrix of chick ovi-

Figure 5

EcoRI and XbaI restriction map of the chicken ovalbumin gene region.

Shaded regions correspond to the leader sequence and 7 exons contained within the ovalbumin gene. EcoRI and XbaI cleavage sites are indicated (adapted from reference 40). Also indicated are the genomic sequences contained within pOv-1, pOv-4, pOv-6, and pOv-7.

duct cells, we prepared nuclear matrices from the oviducts whose transcription parameters are listed in Table 2. A filter containing 1.5 ug of matrix DNA from stimulated, withdrawn and restimulated chick oviducts and various amounts of EcoRI cleaved total nuclear DNA was prepared. This filter was then sequentially hybridized to a series of probes. Autoradiograms of this filter showed that 1.5 ug of the matrix DNA samples from estrogen stimulated (lane S) and restimulated (lane R) chick oviducts contained proportionately more ovalbumin gene sequences than an equivalent amount of total nuclear DNA (Fig. 4A and B). The 1.8 and 2.4 kb fragments containing the central region of the ovalbumin gene were slightly less than 2-fold enriched in the estrogen stimulated chick oviduct matrix DNA samples, while the 9.5 and 14.0 kb fragments, which respectively contained the 3' and 5' ends of the ovalbumin gene, were more than 3-fold enriched. In contrast, a very different pattern was seen with the estrogen withdrawn matrix DNA sample (lane W). In this case, the 1.8 and 2.4 kb fragments were actually depleted in the matrix DNA, while the 9.5 and 14.0 kb fragments were 1.5- to 2-fold enriched (range of 3 experiments). Very similar results were obtained when the matrix DNA preparations were not redigested with EcoRI after DNA purification (not shown). Hence, a substantial change in the relative content of ovalbumin gene sequences associated with the nuclear matrix occurred following hormonal withdrawal.

Other genes provided controls for this analysis. As a positive control, the conalbumin gene was examined. Following estrogen withdrawal, the conalbumin gene was still transcribed at a significant rate, although not as high a rate as before withdrawal (Table 2). Analysis of the same matrix DNA samples examined in Figure 4A and B revealed little change in the content of conalbumin gene sequences during hormonal withdrawal and restimulation (Figure 4D). The 11 kb fragment which contained the 3' end of the gene was

more than 3-fold enriched in the matrix DNA samples, while the 4.0 and 2.5 kb fragments containing 5' and central regions of the conalbumin gene were only slightly enriched. These relative enrichments changed very little during the process of hormonal stimulation, withdrawal, and restimulation. In particular, the 11.0 kb fragment remained highly enriched during hormonal withdrawal. As a negative control, the same filter was rehybridized to a probe detecting the non-transcribed beta-globin gene (Fig. 4C). This gene was not enriched, and in fact, was somewhat depleted relative to total nuclear DNA, in all oviduct matrix DNA samples, in a fashion that was independent of the chicks' hormonal status.

DISCUSSION

Using restriction endonucleases to cleave DNA loops that are anchored to the nuclear matrix we have shown that transcribed genes were preferentially associated with the nuclear matrix of both hen and chick oviduct cells. This was in agreement with results previously obtained when DNase I was used to cleave the matrix-attached DNA loops¹³. If all copies of a given transcriptionally active gene were matrix-associated, then a matrix preparation containing 8% of total nuclear DNA after EcoRI or DNase I cleavage should theoretically be 12.5-fold enriched for the gene while the supernatant DNA should be fully depleted. However, in 15 separate preparations from hen oviduct, (6 with DNase I, 9 with restriction endonucleases) we found that enrichment levels ranged from 3.0 to 6.5-fold. This enrichment accounted for a maximum of 50% of total ovalbumin gene sequences in the matrix DNA fraction while the rest of the ovalbumin genes were always found in the supernatant. Several explanations could account for this loss of ovalbumin gene sequences from the nuclear matrix DNA. First, not all cells of the hen or chick oviduct are tubular gland cells, the cells which transcribe the ovalbumin and conalbumin genes. Tubular gland cells comprise approximately 80% of the cells in the egg-laying hen oviduct and approximately 50-60% of the cells in the estrogen stimulated chick oviduct²⁹. It is notable in this regard that the enrichment for ovalbumin gene fragments, (especially those of low molecular weight) was generally higher in the laying hen than in the hormonally stimulated chick (cf. Fig. 1 and Fig. 4). Second, some of the nuclear matrices probably did not remain stable during the entire isolation procedure. This is difficult to quantitate, since we have been unable to uniformly suspend pelleted matrices for the purpose of counting them. If structures were morphologically disrupted so that they could not pellet

during centrifugation (by shear forces, proteolysis, etc.), then the transcribed genes that were associated with these structures would have been recovered from the supernatant rather than the pelleted (matrix-associated) DNA fraction. Third, it is possible that specific nuclear matrix DNA attachments were somewhat labile under our isolation conditions. This last point may explain the observation that different fragments of the ovalbumin and conalbumin genes appeared to be enriched to different extents in the same matrix DNA sample. Larger DNA fragments, which were more likely to contain a greater number of matrix attachment sites, often appeared to be enriched to higher extents in samples from both hormonally stimulated and withdrawn animals (see Fig. 4, for example).

Similar quantitation problems were encountered in the analysis of non-transcribed genes. Although the results provided here and elsewhere (see Introduction) clearly demonstrate the non-random arrangement of transcribed genes with respect to the nuclear matrix, the arrangement of non-transcribed genes is much less clear. In the chicken oviduct and brain, inactive genes were often only slightly depleted in the DNA associated with the nuclear matrix. In reconstruction experiments, it has been found that a small amount (0.2-4.0%) of exogenously added DNA binds to nuclear matrix preparations³⁰⁻³². A similar amount of non-specific adsorption of detached DNA sequences possibly occurred during our isolation procedure. These adsorbed sequences could significantly alter the depletions that might otherwise have been seen for inactive genes. Presently, however, we cannot rule out the possibility that many inactive genes are arranged randomly with respect to DNA loops and the nuclear matrix.

To analyze the nuclear matrix association of the ovalbumin gene in more detail, hen oviduct nuclear matrices were prepared using XbaI. This restriction endonuclease cleaved the ovalbumin gene and its surrounding sequences into a series of 2.5 to 5 kb fragments that could be detected by cDNA or genomic probes. These fragments were found to be independently attached to the nuclear matrix, since they were enriched in matrix DNA preparations that were not redigested with XbaI after DNA purification. This result was important for three reasons. First, it indicated that the ovalbumin gene contained multiple nuclear matrix attachment sites that were situated in different regions of the gene. This ruled out the possibility that the ovalbumin gene was only attached to the nuclear matrix at a single site such as the promoter region. Second, it showed that transcribed sequences were not associated with the nuclear matrix because of protection from the action

of restriction endonucleases. Active genes in matrix-halo structures were also not resistant to DNase I digestion, as assessed by size analysis of DNase I cleaved matrix DNA (S. Robinson, B. Nelkin, and B. Vogelstein, unpublished data). Finally, since one of the matrix-associated fragments surrounding the ovalbumin gene was outside of the transcription unit itself, it is unlikely that salt-resistant components of the transcriptional apparatus were solely mediating the nuclear matrix association of transcribed genes by specific attachment or nonspecific adsorption. This last point is consistent with the fact that exhaustive ribonuclease digestion of nuclei and nuclear matrices did not disrupt the attachment of the ovalbumin gene or other transcriptionally active genes to the nuclear matrix (Robinson *et al.*, manuscript in preparation).

To determine the reversibility of nuclear matrix gene attachments, nuclear matrices were isolated from the oviduct cells of chicks that had been hormonally manipulated. Following estrogen withdrawal, when the ovalbumin gene was transcribed at a very low rate, fragments of the ovalbumin gene were significantly less enriched in chick oviduct matrix DNA samples than before withdrawal. One explanation of this result is that a fraction of the tubular gland cells in 48-hour hormonally withdrawn chick oviducts was completely inactivated for ovalbumin gene transcription, while the remaining tubular gland cells continued to transcribe the ovalbumin gene at a low rate. It should be noted that the measured transcriptional rate of withdrawn oviduct nuclei never decreased to the background level. The fraction of cells which continued transcription at a low rate could have accounted for the slight enrichment of some ovalbumin gene fragments even during withdrawal. Nevertheless, the results presented in Figure 4 showed that in many of the cells, the matrix attachment of the ovalbumin gene was reversible upon hormonal manipulation. Whether the detachment was due to the loss of estrogen receptors from the nucleus, the cessation of transcription *per se*, or other factors, cannot be answered at this time. It is important to note in this regard that simple cessation of transcription (induced via actinomycin D treatment or heat shock) did not result in the detachment of transcribed sequences from the nuclear matrix (reference 33 and Robinson *et al.*, manuscript in preparation).

What mediates the association between egg white protein genes and the nuclear matrix? One possibility is that the association is mediated at least in part by steroid hormone receptors. Several studies have shown that a significant portion of nuclear hormone receptors are specifically bound to

the nuclear matrix³⁴⁻³⁶. Most of the receptors disappear from the chick oviduct cell nucleus following hormonal withdrawal^{29,37}, as does much of the ovalbumin gene matrix attachment. Several studies have shown that egg white protein genes contain a series of short, repeated sequences (both within and outside of coding sequences) that specifically bind purified hormone receptors^{38,39}. This may be related to the fact that the ovalbumin gene contains several independent matrix binding sites, within and flanking the gene. It is thus possible to speculate that the colocalization of hormone receptors and egg white protein genes on the nuclear matrix is not fortuitous. Obviously, more work is required, on both the actual DNA sequences bound to the nuclear matrix and the proteins mediating this binding, before this speculation can be verified.

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REFERENCES

1. Cook, P.R. and Brazell, I.A. (1975) *J. Cell. Sci.* 19, 261-279.
2. Benyajati, C. and Worcel, A. (1976) *Cell* 9, 393-407.
3. Paulson, J.R. and Laemmli, U.K. (1977) *Cell* 12, 817-828.
4. Vogelstein, B., Pardoll, D.M., and Coffey, D.S. (1980) *Cell* 22, 79-85.
5. Agutter, P.S. and Richardson, J.C. (1980) *J. Cell. Sci.* 44, 395-435.
6. McCready, S.J., A. Krigg, A., and Cook, P.R. (1979) *J. Cell Sci.* 39, 53-62.
7. Lebkowski, J.S. and Laemmli, U.K. (1982) *J. Mol. Biol.* 156, 309-324.
8. Small, D., Nelkin, B., and Vogelstein, B. (1982) *Proc. Natl. Acad. Sci. USA* 79, 5911-5915.
9. Pardoll, D.M. and Vogelstein, B. (1980) *Exp. Cell Res.* 128, 466-470.
10. Nelkin, B.D., Pardoll, D.M. and Vogelstein, B. (1980) *Nuc. Acids Res.* 8, 2895-2908.
11. Jackson, D.A., McCready, S.J., and Cook, P.R. (1981) *Nature* 292, 552-555.
12. Cook, P.R., Lang, J., Hayday, A., Lania, L., Fried, M., Chiswell, D.J., and Wyke, J.A. (1982) *EMBO J* 4, 447-452.
13. Robinson, S.I., Nelkin, B.D. and Vogelstein, B. (1982) *Cell* 28, 99-106.
14. Kaufmann, S.H., Coffey, D.S. and Shaper, J.H. (1981) *Exp. Cell Res.* 132, 105-123.

15. Gross-Bellard, N., Oudet, P., and Chambon, P. (1973) *Eur. J. Biochem.* 36, 32-38.
16. Brunk, C.F., Jones, K.C. and James, T.W. (1979) *Anal. Biochem.* 92, 497-500.
17. Peden, K., Mounts, P., and Hayward, G.S. (1982) *Cell* 31, 71-80.
18. Rigby, P.W.J., Dieckmann, M., Rhodes, C., and Berg, P. (1977) *J. Mol. Biol.* 113, 237-251.
19. Feinberg, A.P. and Vogelstein, B. (1983) *Anal. Biochem.*, in press.
20. Parada, L.F., Tabin, C.J., Shih, C., and Weinberg, R.A. (1982) *Nature* 297, 474-478.
21. Wickens, M.P., Buell, G.N., Crouse, G.F., Carbon, J., and Schimke, R.T. (1979) *Gene* 5, 19-43.
22. Salser, W.A., Cummings, I., Liu, A., Strommer, J., Padayatty, J., and Clark P. (1979) in *Cellular and Molecular Basis of Hb Switching*, Stamatoyannopoulos, G. and Nunhuis, A. Eds., pp. 621-643, Grune and Stratton, New York.
23. Cochet, M., Gannon, F., Hen, R., Marateaux, L., Perrin, F. and Chambon, P. (1979) *Nature* 282, 567-574.
24. Wiskocil, R., Goldman, P. and Deeley, R.G. (1982) *J. Biol. Chem.* 256, 9662-9667.
25. Humphries, P., Cochet, M., Krust, A., Gerlinger, P., Kourilsky, P. and Camban, P. (1977) *Nuc. Acids. Res.* 4, 2389-2406.
26. McKnight, G.S. (1978) *Cell* 14, 403-413.
27. Evans, M.I., Hager, L.J. and McKnight, G.S. (1981) *Cell* 25, 187-193.
28. Darnell, J.E. (1982) *Nature* 297, 365-371.
29. Shepherd, J.H., Mulvihill, E.R., Thomas, P.S., and Palmiter, R.D. (1980) *J. Cell Biol.* 87, 142-151.
30. Pardoll, D., Vogelstein, B., and Coffey, D.S. (1980) *Cell* 19, 527.
31. Berezney, R. and Buchholtz, C.A. (1981) *Exp. Cell Res.* 132, 1-13.
32. Vogelstein, B., Nelkin, B., Pardoll, D., and Hunt, B.F. (1982) *The Nuclear Envelope and the Nuclear Matrix*, G. Maul, ed., pp. 169-181, Alan R. Liss, New York.
33. Small, D., Nelkin, B., Gilmour, D., Lis, J., and Vogelstein, B. (1982) *J. Cell Biol.* 95, 76a.
34. Barrack, E.R., Hawkins, E.F., Allen, S.L., Hicks, L.L., and Coffey, D.S. (1977) *Biochem. Biophys. Res. Commun.* 79, 829-836.
35. Agutter, P.S. and Birchall, K. (1979) *Exp. Cell Res.* 124, 453-460.
36. Barrack, E.R. and Coffey, D.S. (1980) *J. Biol. Chem.* 255, 7265-7275.
37. Mulvihill, E.R. and Palmiter, R.D. (1977) *J. Biol. Chem.* 252, 2060-2068.
38. Mulvihill, E.R., LePennec, J-P., and Chambon, P. (1982) *Cell* 28, 621-632.
39. Compton, J.G., Schrader, W.T. and O'Malley, B.W. (1982) *Biochem. Biophys. Res. Commun.* 105, 96-104.
40. Royal, A., Garapin, A., Cami, B., Perrin, F., Mandell, J.L., DeMeur, M., Bregegere, F., Gannon, F., LePennec, J.P., Chambon, P., and Kourilsky, P. (1979) *Nature* 279, 125-132.