Multiple Structural Features Are Responsible for the Nuclease Sensitivity of the Active Ovalbumin Gene*

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The ovalbumin gene in chick oviduct nuclei or nucleosomes is digested preferentially by either DNase I or staphylococcal nuclease. Staphylococcal nuclease preferentially cuts between and within core particles of the oviduct ovalbumin gene; thus, the ovalbumin gene is more quickly degraded to mononucleosomes and the DNA within these monomers is digested to a nonhybridizable size significantly faster than the chicken globin gene. Mono- and oligonucleosomes generated by partial staphylococcal nuclease digestion at 0°C, but not at 37°C, retain equal sensitivity to DNase I. Most of this sensitivity persists when histone H1 and most of the non-histone chromosomal proteins are removed with 0.6 M NaCl. On the basis of these observations, we propose that nuclease sensitivity of the oviduct ovalbumin gene is due to covalent modifications of the core histones and that this sensitivity is amplified by interaction of other chromosomal proteins with these modified histones.

The chicken oviduct is a widely studied model system for the understanding of the control of eukaryotic gene expression and the mechanism of steroid hormone action. It is now clear that a significant fraction of the regulation of the synthesis of egg white proteins occurs at the transcriptional level (1–3). Thus, detailed molecular knowledge of the chromatin organization of these genes, in both static and dynamic terms, will be essential for an understanding of both gene commitment (the process by which a gene becomes capable of responding to cellular stimuli) and gene activation by steroid hormone receptors.

The nucleosome model of chromatin organization has stimulated investigation of the structural features that distinguish active and inactive chromatin. From the work of a number of groups, it has become clear that transcriptionally active DNA sequences are present in nucleosomal structures that appear to be similar to those associated with most inactive DNA (4–7). However, these nucleosomes are somehow different from the majority population since the active gene DNA sequences contained in them are unusually sensitive to digestion by exogeneous nucleases. Active chicken globin (8) and ovalbumin (9) genes and most other active genes (10, 11) in a variety of organisms are highly sensitive to digestion by DNase I, and some active genes, such as those coding for ribosomal RNA sequences (12–15) and integrated mouse mammary tumor virus (16), also show an increased sensitivity to staphylococcal nuclease. Our present understanding of the particular features responsible for this nuclease sensitivity is limited. Elucidation of the molecular basis of the nuclease sensitivity of active genes should shed light on questions relating to the relative roles of histones and their modifications versus non-histone chromosomal proteins (NHCP) in cell differentiation and transcriptional activation of genes, and provide a foundation for future studies on the nature of the interaction of protein complexes such as RNA polymerases and hormone receptors with specific sites in chromatin.

Garel and Axel (9) found that ovalbumin gene sequences in isolated nucleosomal monomers are not preferentially digested by DNase I, suggesting that some higher order aspect of chromatin structure is required for this nuclease sensitivity. We therefore set out to ask how large chromatin fragments must be to retain sensitivity to DNase I, to identify some factor or structural aspect of ovalbumin gene chromatin that is responsible for this sensitivity, and to investigate the relative importance of histones and NHCPs for DNase I sensitivity. We found that monomer nucleosomes can be prepared that retain at least partial DNase I sensitivity; thus, this property is largely dependent on the structure and composition of individual nucleosomes. Furthermore, salt-extracted nuclei and oligo- and mononucleosomes retain some of the relative DNase I sensitivity of the ovalbumin gene. We also characterized the digestion of ovalbumin gene chromatin by staphylococcal nuclease, another enzyme that digests chromatin in a gene-specific manner; early in digestion, ovalbumin nucleosomes are rapidly released from larger chromatin structures, and in the limit digest ovalbumin gene sequences are depleted.

MATERIALS AND METHODS

Preparation of Nuclei—Nuclei were isolated from the oviduct magnum or liver of egg-laying White Leghorn hens essentially as described by Mulvihill and Palmiter (17). Nuclei were stored in Buffer NC (50 mM Tris, pH 8.2, 5 mM Mg(CH3COO)2, 35% glycerol, 0.5 mM dithiothreitol) at −20°C; nuclease sensitivity was stable for several weeks.

Nuclease Digestion of Nuclei and Chromatin—Several different procedures were used for the digestion of nuclei and of chromatin fractions with staphylococcal nuclease and DNase I. The digestion times and amounts indicated are only approximate; the exact conditions for a particular experiment were dependent on the batch of nuclease, the preparation of nuclei or chromatin, and the extent of digestion desired, and so were often determined in pilot experiments. Before digestion, nuclei were washed two or three times by centrifugation and re-suspension in either RS buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM Mg(CH3COO)2) for DNase I experiments, or in Buffer NC for staphylococcal nuclease experiments.

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1 The abbreviations used are: NHCP, non-histone chromosomal protein; HMG, high mobility group protein; EGTA, ethylene glycol bis[β-aminoethoxy]ethyl)N,N',N''-tetraacetic acid; ov, ovalbumin; gb, globin.
For determination of the sensitivity of the ovalbumin gene to staphylococcal nuclease, nuclei were suspended in SN buffer (10 mM Tris, pH 7.9, 0.1 mM CaCl2, 1.5% sodium dodecyl sulfate at ~0.5 mM of DNA/ml, warmed to 37°C, and 20 units/ml of staphylococcal nuclease (Worthington, NJ) was added. Aliquots were removed from the digestion mixture after various times (up to 90 min) and digestion was terminated by adding EDTA to a final concentration of 5 mM. Partial staphylococcal nuclease digests for isolation of oligonucleosomes were generally performed on ice. Nuclei were resuspended in SN buffer at 0.5 to 1.0 mg of DNA/ml and 100 units/ml of staphylococcal nuclease was added. Digestion times ranged from 90 to 210 min. In other cases, partial staphylococcal nuclease digests were performed at 4°C using 1 to 5 units/ml, with digestion proceeding for 10 to 15 min.

DNase I digestion of nuclei was performed at 37°C. Nuclei were suspended in RS buffer at approximately 0.5 mg of DNA/ml and 5 to 10 μg/ml of DNase I (Worthington, PFF) was added. Kinetic experiments included times of up to 1 h, but generally 15 min of digestion was sufficient to demonstrate maximum sensitivity of the ovalbumin gene. Digestion was terminated by addition of EDTA to 5 mM. For DNase I digestion of staphylococcal nuclease-treated chromatin fractions, the chromatin was first dialyzed at 4°C into RS buffer containing 10 μg/ml EDTA (to ensure complete inactivation of any remaining nuclease). 2 to 5 μg/ml of DNase I and 0.2 to 0.3 mg/ml CaCl2 were then added, and samples were incubated at 37°C for 10 to 15 min.

The fraction of DNA rendered soluble by nuclease digestion was determined by a phenolamine assay (18).

Sucrose Gradient Centrifugation of Staphylococcal Nuclease Digests—Staphylococcal nuclease digests were fractionated by centrifugation in linear 5 to 20% sucrose gradients containing 5 mM EDTA, pH 7.0; some gradients also contained 0.6 M NaCl. Gradients were centrifuged for 15 min at 750 × g. Nuclear pellets were resuspended in 4 ml of RS buffer containing 55% glycerol (RS buffer/glycerol), and to 600 μM NaCl. After 1 h of incubation on ice with occasional mixing, samples were centrifuged for 15 min at 750 × g. Nuclear pellets were resuspended in 4 ml of RS buffer/glycerol plus NaCl, incubated another 30 min on ice, and repelleted. All samples were washed twice in 4 ml of RS buffer/glycerol without added NaCl and recentrifuged. Finally, they were resuspended in 2 ml of RS buffer/glycerol for digestion with DNase I. A similar procedure was performed on ice with 300 mM NaCl to yield opaque pellets that were readily resuspended. At higher NaCl concentrations the nuclei formed an almost clear, rather than cloudy suspension and after centrifugation the pellet was transulent and less compact than normal. However, it was possible to resuspend this material with gentle mixing. These salt-extracted nuclei did not have the high viscosity characteristic of lysed nuclei. Use of round-bottomed plastic tubes allowed efficient resuspension of salt-extracted nuclei.

RESULTS

Sensitivity of Ov/Gb Sequences to Staphylococcal Nuclease—Fig. 1 shows that staphylococcal nuclease preferentially degrades ovalbumin gene sequences in nuclei. DNA was isolated from hen oviduct nuclei before and after digestion with staphylococcal nuclease; three different amounts of each sample were hybridized to cDNAo, and cDNAg. The globin probe was used as a representative nonexpressed oviduct gene, and as an internal control to demonstrate that each DNA sample was capable of normal hybridization. DNA from undigested nuclei hybridized nearly identically with ovalbumin and globin cDNAs; the ratio Ov/Gb of the slopes of the two lines is 0.87. After digestion with staphylococcal nuclease, an Ov/Gb ratio of 0.54 was obtained.

Fig. 24 shows the time course of digestion of oviduct nuclei with staphylococcal nuclease. Each sample time was assayed for the fraction of DNA rendered acid-soluble and the nucleo-resistant DNA was hybridized to cDNAo and cDNAg. Each point represents an average hybridization value (or ratio of two such values) calculated as shown in Fig. 1. In the limit digest, 50 to 55% of the input DNA was rendered acid-soluble. At this point, the Ov/Gb ratio is about half of the starting value, due largely to a decrease in cDNAo hybridization, although there is also an increase in cDNAg hybridization.

![Fig. 1](image-url)
The decrease in cDNA\textsubscript{w} hybridization, and in the Ov/Gb ratio, occurs throughout the time course of digestion at a nearly constant rate relative to the overall acid solubilization of DNA. Preferential digestion of ovalbumin sequences must continue after chromatin has been converted to mononucleosomes, which is achieved when 15 to 20\% of the DNA is solubilized.

This sensitivity of the ovalbumin gene to staphylococcal nuclease is oviduct-specific; digestion of liver nuclei with staphylococcal nuclease (Fig. 2B) results in a much smaller change in the Ov/Gb ratio. In this tissue, the ovalbumin gene is no more sensitive to staphylococcal nuclease than are bulk DNA sequences; the small decrease in the Ov/Gb ratio is due to the higher than average resistance of the globin genes to staphylococcal nuclease seen in both tissues. The tissue specificity of this effect is more apparent in a plot of the oviduct/liver ratio of ovalbumin or globin sequences remaining after various extents of staphylococcal nuclease digestion (Fig. 2C). This ratio remains constant for globin, but it decreases progressively for ovalbumin. Thus, the ovalbumin gene is clearly in different states in the oviduct and liver, while the globin genes, which are inactive in both tissues, display the same degree of sensitivity to staphylococcal nuclease in oviduct and liver.

Digestion of nuclei or chromatin with staphylococcal nuclease (or DNase I; see below) results in a much smaller decrease in the amount of all DNA sequences. However, some sequences are digested at an especially high rate, so that their relative concentration in the product also decreases significantly. It is this relative effect, specific to only a subset of all DNA sequences, that we refer to as nuclease (DNase I or staphylococcal) sensitivity.

In apparent contradiction to our findings, Garel and Axel (9) reported that mononucleosomes prepared from oviduct nuclei with staphylococcal nuclease contain normal amounts of ovalbumin sequences; thus, they suggested that the ovalbumin gene is not sensitive to staphylococcal nuclease. We therefore digested oviduct nuclei with staphylococcal nuclease at 37\(^\circ\)C until most of the chromatin was converted to mononucleosomes, and separated the products on a sucrose gradient as shown in Fig. 3. Table I presents the amount of cDNA\textsubscript{w} and cDNA\textsubscript{h} hybridization and the Ov/Gb ratio for DNA extracted from undigested nuclei, from the total staphylococcal nuclease digest, and from the mononucleosome fraction of this digest. Comparison of results for the total digest and the undigested nuclei clearly shows the preferential sensitivity of the ovalbumin gene to staphylococcal nuclease described above. However, the mononucleosomal DNA has an Ov/Gb ratio similar to that found in the undigested DNA. Our explanation for these results, and those of Garel and Axel (9), is that the ovalbumin gene sequences are preferentially converted to mononucleosomes and are also further digested to a nonhybridizable form. A balance between these two processes during intermediate stages of digestion maintains a relatively constant Ov/Gb ratio in mononucleosomes, while the ovalbumin gene content and Ov/Gb ratio in oligonucleosomes diminishes.

**Size Distribution of Ovalbumin Sequences in Nucleosomes Generated by Partial Staphylococcal Nuclease Digestion—** To investigate the size distribution of specific sequences during staphylococcal nuclease digestion, nuclei were partially digested at 0\(^\circ\)C to yield a broad distribution of oligonucleosome sizes. At 0\(^\circ\)C, digestion is limited to clipping linker regions between nucleosomes, while trimming and cutting within nucleosomes is suppressed (22), thus minimizing destruction of ovalbumin sequences. In these experiments, we also determined the effects of fractionating the chromatin in gradients containing 0.6 \text{ M NaCl}, which removes histone H1 and a significant fraction of the NHCP.

Visual inspection of the absorption profiles (Fig. 4, A and B) from experiments where portions of a single digest were

**Fig. 3. Isolation of mononucleosomes.** Hen oviduct nuclei were digested with staphylococcal nuclease at 37\(^\circ\)C. A portion of this digest was fractionated by sucrose gradient centrifugation and the mononucleosome fraction pooled as shown.

**Table I**

<table>
<thead>
<tr>
<th></th>
<th>Ovalbumin</th>
<th>Globin</th>
<th>Ov/Gb</th>
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<tr>
<td>% hybrid/\mu g DNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undigested nuclei</td>
<td>3.03</td>
<td>3.34</td>
<td>0.91</td>
</tr>
<tr>
<td>Total digest</td>
<td>2.45</td>
<td>3.89</td>
<td>0.65</td>
</tr>
<tr>
<td>Mononucleosomes</td>
<td>3.33</td>
<td>3.74</td>
<td>0.94</td>
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fractionated plus and minus salt leads to several observations. Nucleosomes sediment more slowly in the salt gradients, and they are more poorly resolved. Salt also alters the relative amounts of various size classes; mononucleosomes and small oligonucleosomes are more abundant and there is a corresponding decrease in the amount of rapidly sedimenting material. Apparently, many of the oligonucleosomes on the native gradients do not contain a continuous DNA molecule but are instead held together by a salt-dissociable interaction.

Fractions from these gradients were pooled, and the distributions of ovalbumin and globin sequences, expressed as Ov/Gb, across these gradients were determined (Fig. 4, histograms). In both cases, the mononucleosome fraction is significantly enriched in ovalbumin sequences relative to globin sequences. The gradient of ovalbumin gene enrichment is much steeper when the digest is fractionated in the presence of salt. The salt-washed monomers have a significantly higher Ov/Gb ratio than do the native monomers (due to a higher enrichment for ovalbumin sequences), while the material at the bottom of the salt gradient is even more depleted in ovalbumin gene sequences than the material at the bottom of the native gradient. This indicates that the internucleosomal association disrupted by 0.6 M NaCl is more prevalent in ovalbumin gene chromatin than in globin gene or total chromatin from the oviduct.

While a similar effect of salt on the sedimentation profiles of oligonucleosomes generated by partial staphylococcal nuclease digestion is also seen with liver nuclei, the distribution of ovalbumin sequences is significantly different. In liver digests, the Ov/Gb ratio is essentially constant across the gradient. Both ovalbumin and globin are depleted in mononucleosomes and enriched in the larger fractions, following the pattern that was found for the globin genes in the oviduct (data not shown). Thus, the way in which the ovalbumin gene fractionates in such experiments appears to be associated with the differentiated state of the gene.

**DNase I Sensitivity of Nuclei**—The ovalbumin gene in hen oviduct nuclei is sensitive to digestion by DNase I (9). The kinetics of this digestion is shown in Fig. 5. Initially, ovalbumin sequences decrease rapidly; a plateau value (about 40% of the starting value) is reached when 15 to 20% of the DNA has become acid-soluble. The amount of hybridization to cDNA

![Fig. 5. DNase I digestion of hen oviduct nuclei.](image)

**Fig. 5. DNase I digestion of hen oviduct nuclei.** Hen oviduct nuclei were suspended in RS buffer at 37°C and DNase I was added. Samples were removed after various times of digestion up to 25 min. The amount of DNA rendered acid-soluble in each sample was measured. DNA from each sample was hybridized to cDNA

![Fig. 6. DNase I digestion of hen oviduct chromatin.](image)

**Fig. 6. DNase I digestion of hen oviduct chromatin.** Hen oviduct nuclei were first briefly digested with staphylococcal nuclease at 37°C, lysed, and dialyzed into RS buffer. The resulting crude chromatin preparation was then digested with DNase I for various lengths of time. Acid solubilization of each sample was measured. DNA was isolated and hybridized to cDNA

DNase I Sensitivity of Chromatin—To identify structural features of chromatin responsible for the DNase I sensitivity of the ovalbumin gene, a method of chromatin isolation that does not destroy DNase I sensitivity was needed. Various methods of chromatin isolation involving lysis of nuclei followed by centrifugation and homogenization of the resulting chromatin gel consistently resulted in the loss of DNase I sensitivity. Similar treatments have previously been shown to disrupt the repeating nucleosomal structure of chromatin. However, brief treatment of nuclei with staphylococcal nuclease before lysis eliminates the need for mechanical shearing, and the repeat structure of the chromatin is retained (23). Fig. 6 shows the kinetics of DNase I digestion of oviduct

![Fig. 4. Fractionation of native and salt-extracted partial staphylococcal nuclease digests of oviduct nuclei.](image)

**Fig. 4. Fractionation of native and salt-extracted partial staphylococcal nuclease digests of oviduct nuclei.** Hen oviduct nuclei were partially digested with staphylococcal nuclease on ice. To half of the digest NaCl was added to a concentration of 0.6 M. The digests were then loaded on sucrose gradients without (A) or with (B) 0.6 M NaCl, centrifuged, and fractionated as shown. DNA was isolated from each fraction and assayed for the content of ovalbumin and globin sequences. Histograms show the hybridization results (Ov/Gb) for the material collected from each fraction. The total unfractonated digest gave an Ov/Gb ratio of 1.09.

Remains essentially constant throughout the time course of digestion. Thus, the Ov/Gb ratio, which also decreases rapidly at first and then plateaus at a level of around 40% of the starting value, provides an equivalent measure of the DNase I sensitivity of the ovalbumin gene.
Nuclease Sensitivity of the Ovalbumin Gene

chromatin that was prepared by a short incubation with staphylococcal nuclease, followed by lysis of the nuclei, and dialysis of the chromatin back into DNase I digestion buffer. The DNase I sensitivity of the ovalbumin gene in such chromatin preparations is similar to that in nuclei. Early in the reaction, the amount of ovalbumin hybridization decreases rapidly; however, rather than reaching a plateau, as in nuclear digestion experiments, cDNA\_\text{ov}, hybridization continues to fall. The amount of cDNA\_\text{ov} hybridization also falls slowly throughout the reaction, again in contrast to the nuclear digestion experiments. The Ov/Gb ratio, however, still plateau when 15 to 20% of the DNA has become acid-soluble. Thus, this digestion process appears to go through two phases. At early times, ovalbumin sequences are rapidly and specifically degraded; at later times, both ovalbumin and globin, and presumably all other DNA sequences are slowly degraded. The plateau in the Ov/Gb ratio reached with chromatin is higher than that attained with nuclei (0.6 \textit{versus} 0.4; compare Figs. 5 and 6).

This experiment demonstrates the utility of the Ov/Gb ratio. DNase I digestion of chromatin preparation does not result in a unique per cent hybridization per \mu g of DNA value for either cDNA; both values continually decrease with larger extents of digestion. However, the Ov/Gb ratio reaches a plateau value that provides a well-defined parameter to characterize a preparation of chromatin or nucleosomes.

To show that this partial loss of DNase I sensitivity is directly due to an effect of staphylococcal nuclease on chromatin structure rather than a result of nuclear lysis or dialysis, the experiment shown in Fig. 7 was performed. Oviduct nuclei were incubated with staphylococcal nuclease for various lengths of time, nuclei were disrupted, dialyzed, and further digested with DNase I. As the extent of staphylococcal nuclease digestion increased, the Ov/Gb ratio in the chromatin preparation decreased, in agreement with the results presented above. Staphylococcal nuclease digestion followed by DNase I digestion actually results in a higher final Ov/Gb ratio than does DNase I digestion alone. Hence, the DNase I sensitivity, which is defined as the ratio of the two curves, changes from an initial value of 0.45 with no staphylococcal nuclease digestion to a final value of 1.0 at the point where 20% of the DNA has become acid-soluble.

**Digestion of Nucleosomes with DNase I**—To determine the size of the smallest unit of chromatin that retains sensitivity to DNase I, mono- and oligonucleosomes were prepared by digestion of nuclei with staphylococcal nuclease in two different ways.

In the first approach, nuclei were digested with staphylococcal nuclease at 37°C until about 20% of the DNA had become acid-soluble and most of the chromatin had been converted into mononucleosome cores. Part of the digest was fractionated on sucrose gradients to isolate the monomers (similar to Fig. 3). Portions of the isolated monomers and of the total digest were then redigested with DNase I. In neither case was there any change in the Ov/Gb ratio (Table II). Thus, in agreement with Garel and Axel (9), we observe that the ovalbumin sequences in core mononucleosomes show no specific sensitivity to DNase I. These results are also consistent with those of the previous experiment.

For the second approach, staphylococcal nuclease digestion was performed at 6°C to retard trimming and nicking of nucleosomes (22). This generated a mixture of particles, ranging from monomers to large multimers, which were separated on sucrose gradients (Fig. 8A). Half of each fraction was then further digested with DNase I. The Ov/Gb ratio for each fraction before and after DNase I digestion is shown, along with the digested/undigested ratio, which is a measure of the sensitivity to DNase I. All size fractions, ranging from monomers to large oligonucleosomes (n > 10), retain a partial DNase I sensitivity of about 0.75. Nuclei show a sensitivity of about 0.40 (Fig. 5), while a completely insensitive preparation would be characterized by a ratio of 1.0.

To ask whether histone H1 and NHCPs are necessary for the DNase I sensitivity of such nucleosomal particles, a portion of the staphylococcal nuclease digest was fractionated on sucrose gradients containing 0.6 M NaCl and various size classes of these salt-washed oligonucleosomes were digested with DNase I (Fig. 8B). These salt-washed nucleosomes also retain some DNase I sensitivity, although slightly less than the native nucleosomes. Again, there is little correlation between the oligomeric size and the level of sensitivity. The fraction containing the very largest oligomers consistently shows no sensitivity of ovalbumin sequences to DNase I, but these fractions contain such low amounts of ovalbumin DNA that these values may not be significant.

**Salt Extraction of Nuclei**—The previous experiments suggest that at least some of the DNase I sensitivity of the ovalbumin gene is retained in the presence of 0.6 M NaCl. However, since staphylococcal nuclease had already destroyed much of the DNase I sensitivity of the gene in those experiments, it was necessary to utilize a more effective method for retaining nucleosome structure. The procedure used was to dialyze nuclei in 0.2 M NaCl at 4°C for 5 hours, followed by dialysis into cold 0.1 M Tris-HCl, pH 7.5, containing 0.1 M NaCl and 0.01 M EDTA. The DNA was then sheared to about 500 nm in a glass homogenizer, cooled on ice, and then fractionated on sucrose gradients. The oligonucleosomes were then isolated by dialysis into sucrose buffer and dialysis into DNase I digestion buffer.

**TABLE II**

<table>
<thead>
<tr>
<th>Digestion of core mononucleosomes with DNase I</th>
<th></th>
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<tbody>
<tr>
<td>Total digest</td>
<td>0.93</td>
</tr>
<tr>
<td>Mononucleosomes</td>
<td>1.01</td>
</tr>
<tr>
<td>DNase I sensitivity</td>
<td></td>
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<tr>
<td>\textit{versus} to Ov/Gb.</td>
<td>0.93</td>
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</table>

FIG. 7. The effect of the extent of staphylococcal nuclease digestion on subsequent DNase I sensitivity. Oviduct nuclei were suspended in SN buffer and digested with staphylococcal nuclease at 37°C for times of up to 135 min. A small sample from each time point was assayed for the amount of DNA made acid-soluble (abscess). The remainder of each sample was dialyzed into RS buffer + EGTA, and half was then further digested with DNase I. DNA from samples digested only with staphylococcal nuclease (C --- C) and further digested with DNase I (\(\Delta\) --- \(\Delta\)) were assayed for hybridization to cDNA\_\text{ov} and to cDNA\_\text{ov} the ratio of hybridization to the two probes Ov/Gb is plotted. DNase I sensitivity is \(\bullet\) for each sample pair is the ratio of these values, digested/undigested. The first time point (0% digestion) is for nuclei that were directly digested with DNase I in RS buffer.
ments, the role of salt-extractable components in DNase I sensitivity could not be accurately assessed. In the experiments shown in Fig. 9, nuclei were exposed to concentrations of NaCl up to 0.6 M in DNase I digestion buffer containing 35% glycerol, and then they were washed with buffer without NaCl. Addition of 35% glycerol stabilizes the nuclei during salt-extraction and washing procedures (see “Materials and Methods”). Nuclei treated with up to 0.3 M NaCl had DNase I sensitivity levels ranging from 0.45 to 0.65. After washing with 0.4 to 0.6 M NaCl, DNase I sensitivity was reduced (0.75 to 0.90) but not eliminated. There was some nuclear disruption during the centrifugation steps, which accounts for the rather high Ov/Gb ratio for nuclei treated only with low salt and for the variability between samples seen in these experiments; nevertheless, it is clear that the Ov/Gb ratios for all samples extracted with low salt are lower than for samples extracted with high salt, and that some critical component is extracted between 0.3 and 0.4 M NaCl.

**DISCUSSION**

In the experiments presented here, we have investigated the sensitivity of the ovalbumin gene to DNase I and staphylococcal nuclease. Since nucleosomes have been shown to be associated with actively transcribed genes in a number of systems (4-9), the goal of these studies was to use nucleases to probe the nature of variations in nucleosomal structure and organization that distinguish active ovalbumin gene chromatin from the much larger amounts of genetically inactive chromatin found in the oviduct tubular gland cell nucleus. Our results indicate that both core nucleosomal structure (probably due to specific histone modifications or substitutions) and extranucleosomal or linker region structure (due to the presence of specific NHCP and/or H1 species) are important for the functional differentiation of the ovalbumin gene.

**Sensitivity of Ovalbumin Gene Chromatin to Staphylococcal Nuclease**—Digestion of nuclei with staphylococcal nuclease involves several types of cutting. First, this nuclease cuts chromatin between nucleosomes, generating oligomers of nucleosomes which are further cut into smaller multimers and mononucleosomes. The intermediates contain pieces of DNA that are integral multiples of 196 base pairs of DNA and a full complement of the core histones and histone H1, and large amounts of NHCP. In the second step, mononucleosomes are further processed to core nucleosomes, which contain ~140 base pairs of DNA and a full complement of the core histones, but which lack H1 and most of the NHCP. Some core nucleosomes are digested further, giving rise to a variety of particles containing less that 140 base pairs of DNA associated with only a partial complement of histones, or with other proteins (25). These events are only partially separated in time. In our experiments we have found evidence for differential sensitivity of the ovalbumin gene to all three processes.

After mild digestion of oviduct nuclei with staphylococcal nuclease and fractionation of the chromatin on sucrose gradients, the smaller oligonucleosome fractions are highly enriched in ovalbumin sequences, and the largest chromatin fragments are correspondingly depleted in ovalbumin DNA (Figs. 4 and 8). The converse applies to globin sequences,
The distribution of nucleosomes on salt-containing gradients (Fig. 4B) probably depends only on the susceptibility of a site(s) within the linker region between nucleosomes to the nuclease, and should thus be a function of the nature of this linker region and/or the way in which the nucleosomal fiber is packed into the chromatin fiber. The Ov/Gb ratio of ~2 found in the mononucleosome fraction on the salt gradients suggests that the linker regions in ovalbumin gene chromatin are about twice as accessible to staphylococcal nuclease as are the linker regions in inactive genes. Bellard et al. (5) found a similar distribution of ovalbumin and globin sequences among DNA bands resulting from partial staphylococcal nuclease digestion when the products were fractionated by polyacrylamide gel electrophoresis.

The slower sedimentation of oligonucleosomes in the salt gradients has been observed before (22, 26) and has been attributed to uncoiling of the chromatin fiber, due to removal of histone H1 and NHCP. Increased density of the gradient medium due to the presence of NaCl would have minimal effect on the sedimentation velocity. The removal of H1 and NHCP also changes the proportions of various nucleosome size classes. It seems likely that in the absence of salt there are oligonucleosomal complexes whose DNA molecules have been cleaved one or more times by the nuclease, but which are held together by protein interactions. The magnitude of this effect is variable; it is especially striking after relatively short extents of digestion.

An enrichment of ovalbumin sequences in mononucleosomes is observed in native gradients, confirming earlier observations (27), but the effect is more striking in the salt gradients. The differences in the distributions of ovalbumin and globin sequences due to the presence of salt suggest that active ovalbumin gene chromatin contains more of these salt-dissociable bridges than does inactive gene chromatin. We estimate that these bridges hold together only 20 to 40% of all staphylococcal nuclease-cleaved internucleosomal linkers, but a majority of cleaved ovalbumin gene linker regions contain such bridges. This property of the ovalbumin gene might reflect a unique class of histone H1 or some NHCP that is enriched in ovalbumin chromatin from the oviduct; for example, it has been suggested that high mobility group (HMG) proteins may replace H1 in the linker region of some nucleosomes and that HMGs are enriched in active gene chromatin (22, 28-31). The higher susceptibility of ovalbumin gene linker regions to staphylococcal nuclease and the higher prevalence of salt-sensitive internucleosomal bridges may both be due to either the same or different factor or structural feature. Artificial aggregation of nucleosomes could also be responsible for the different distributions of oligonucleosomes in native and salt gradients, but it would not explain the different distributions of ovalbumin and globin sequences on these gradients.

As oviduct nuclei are digested by staphylococcal nuclease to the limit product, where approximately 50% of the DNA is rendered acid-soluble, a second type of sensitivity of the ovalbumin gene is observed, resulting in the preferential loss of hybridizable ovalbumin sequences. Much of the decrease in ovalbumin sequences occurs when there are essentially no ovalbumin sequences present in structures larger than mononucleosomes. This implies that staphylococcal nuclease also recognizes some special feature of the core nucleosome structure of ovalbumin chromatin. This activity of staphylococcal nuclease preferentially reduces a significant fraction of ovalbumin DNA sequences to pieces too small to hybridize to cDNA under our conditions, i.e. significantly smaller than the 140-base pair core nucleosome that is the most prominent product in the limit staphylococcal nuclease digest. Other workers (4, 25, 32, 33) have demonstrated that in the limit digestion product the resistant DNA is heterogeneous in size, with products both smaller and larger than 140 base pairs; many of the smaller DNA fragments are present as discrete DNA-protein complexes (25). On the other hand, globin nucleosomes may be inaccessible to digestion beyond the 140-base pair size; 70% of the original globin sequences would then remain in the limit digest (140 base pairs of each original 200-base pair unit). Since only 50% of the total nuclear DNA remains, this would result in the apparent enrichment for globin sequences that we have observed.

Sensitivity to staphylococcal nuclease digestion appears to be a variable feature of active genes. The active chick globin gene is reported to be insensitive to staphylococcal nuclease (8, 34, 35), but Panet and Cedar (16) have described a striking sensitivity of integrated murine leukemia viral DNA sequences to this nuclease. The differences between various active genes that determine their relative sensitivity to staphylococcal nuclease digestion may be subtle. For example, Weintraub and Groudine (8) found that trypsin treatment of reticulocyte nucleosomes allows preferential digestion of globin DNA sequences by staphylococcal nuclease.

The Basis of the DNase I Sensitivity of the Ovalbumin Gene—The original goal of these studies was to determine what aspect of chromatin structure is responsible for the sensitivity of the ovalbumin gene to DNase I. Garel and Axel (9) found that the isolated mononucleosome had lost DNase I sensitivity, suggesting that DNase I sensitivity is due to the higher order folding of the chromatin fiber. However, since digestion of nuclei with staphylococcal nuclease has effects beyond simply unfolding the chromatin fiber, numerous other possibilities remained.

We confirmed the observation of Garel and Axel (9) that core mononucleosomes generated by staphylococcal nuclease at 37°C have lost their DNase I sensitivity (Table 1). Further experimentation (Fig. 7) demonstrated that this result represents the end point of a progressive loss of DNase I sensitivity when chromatin is digested by staphylococcal nuclease. When the products of staphylococcal nuclease digestion at 0°C were examined, DNase I sensitivity was found to be independent of size (Fig. 8). Although the digest as a whole had lost a significant fraction of its original DNase I sensitivity, ovalbumin mononucleosomes are as sensitive to DNase I as are much larger oligonucleosomes. Although some of the DNase I sensitivity of the ovalbumin gene may be due to higher order chromatin structure, there is a component of DNase I sensitivity that is associated with the structure of individual nucleosomes. Since staphylococcal nuclease and DNase I cut within the nucleosome core at different sites (36), the effect of staphylococcal nuclease on DNase I sensitivity may be relatively indirect. Cutting between nucleosomes and destruction of the DNase I-sensitive state are probably independent processes which reach completion at about the same time only by chance.

We also attempted to identify the protein components of ovalbumin chromatin responsible for its DNase I sensitivity. The literature has suggested two possibilities. One viewpoint claims that modification of histones is responsible for this property. For example, it has been shown that nuclei and chromatin containing elevated levels of acetylated histones are rapidly digested by DNase I (37-39). Alternatively, the apparent enrichment of HMGs in active gene chromatin and their rapid release from nuclei by DNase I (28-31, 49) has suggested to other workers that HMGs may be associated with the site that makes active genes especially sensitive to DNase I. Recently, Weisbrod and Wemtraub (41) have showed
by reconstitution experiments that a specific HMG is required for the DNase I sensitivity of the globin gene in chick erythrocyte chromatin. Other types of histone modification and/or specific NHCP could also be important for DNase I sensitivity of active genes.

Significant DNase I sensitivity remains in oligonucleosomes even after extraction of H1 and most NHCP by NaCl (Fig. 8). Similar levels of DNase I sensitivity remain in nuclei after salt extraction (Fig. 9). These results suggest that DNase I recognizes some feature of the core nucleosome of the active ovalbumin gene. This feature is most likely some type of histone modification, although it is also possible that a very tightly bound NHCP is responsible for this property.

The nuclear salt extraction experiments (Fig. 9), on the other hand, also point to a role for a NHCP fraction in DNase I sensitivity, since salt extraction results in a partial loss of DNase I sensitivity. Our experiments are consistent with the suggestion that HMGs are involved in the DNase I sensitivity since 0.35 M NaCl is used to dissociate HMGs (41) while we found the greatest loss of DNase I sensitivity between 0.3 and 0.4 M NaCl. However, a variety of other NHCPs are also extracted from nuclei under these conditions. The salt-extractable factor(s) could affect the DNase I sensitivity of the ovalbumin gene either solely through interactions with individual nucleosomes or by altering the way in which nucleosomes interact with one another. Models have been proposed (42, 43) where activation of genes involves a cooperative propagation of changes in nucleosome structure along the chromatin fiber. Since mononucleosomes retain some of the properties of active gene chromatin, such cooperative changes cannot be the sole feature distinguishing active gene chromatin, but may still play an important role.

As a unifying hypothesis, we suggest that some covalent modification of core histones establishes partial DNase I sensitivity and this effect is then magnified by the recognition of this structural difference by other chromosomal proteins which become associated with these differentiated nucleosomes. The implication is that the core histone modification partially relaxes the nucleosomal structure and that HMGs, a special class of H1, or some other NHCP increase the exposure of the DNA of these modified nucleosomes to nucleases. This hypothesis leaves unanswered the crucial question of how the nucleosomes associated with specific genes become modified during development.

Although the features of ovalbumin gene chromatin that we have described are all oviduct-specific, from these experiments we cannot determine whether they are a consequence of the initial differentiation of oviduct tubular gland cells, or of hormonal stimulation and transcriptional activity. We have previously shown (44, 45) that the ovalbumin gene in the oviduct remains sensitive to DNase I under conditions where it is inactive (<1 transcript/h). We expect that further experiments along these lines will show that some aspects of ovalbumin gene chromatin structure are sensitive to the hormonal state of the tissue while others are not.

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