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A close association between sites of DNase I hypersensitivity and sites of enhanced cleavage by micrococcal nuclease in the 5'-flanking region of the actively transcribed ovalbumin gene

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The organization of chromatin was analysed in a segment of the chicken ovalbumin gene extending 6.5 kb upstream from the start site of transcription. Nuclei of chicken oviduct cells and of erythrocytes, and preparations of 'naked' DNA were digested with DNase I and with micrococcal nuclease. The locations of specific nuclease cleavage sites were determined by analyzing the fragments obtained with an indirect end-labeling technique. In oviduct nuclei there are four regions of DNase I hypersensitivity centered at ~0.15, 0.80, 3.2 and 6.0 kb upstream from the mRNA cap site. DNase I hypersensitive regions are absent from the 5'-flanking regions of erythrocyte nuclei. Micrococcal nuclease cleavage sites were found that are unique to oviduct nuclei and others that are enhanced in oviduct nuclei, relative to erythrocyte nuclei and to naked DNA. The locations of these micrococcal nuclease cleavage sites are closely associated with the DNase I hypersensitive regions. Nuclease hypersensitivity in the 5'-flanking region of oviduct nuclei reflects alterations in chromatin structure that are specifically correlated with gene expression. Our results suggest the presence at hypersensitive regions of specific proteins which alter the chromatin structure, making the DNA more accessible to nuclease attack.
Key words: ovalbumin gene/DNase I hypersensitivity/micrococcal nuclease sensitivity/active chromatin

Introduction

The elucidation of the structure of the chromatin of active genes and their flanking regions is one prerequisite to the ultimate understanding of the control of gene expression in eukaryotes. Considerable information on chromatin structure has been obtained from the analysis of digestion patterns obtained after treatment of nuclei with endonucleases. Chromatin in the active state has an altered structure compared with its inactive counterpart; it is more sensitive to digestion by DNase I (Weintraub and Groudine, 1976) and by micrococcal nuclease (Bellard *et al.*, 1977; Bloom and Anderson, 1982). (For other references see Chambon, 1977; Mathis *et al.*, 1980; Weissbrod, 1982). Within the 5'-flanking region of the active gene, localized regions called hypersensitive sites show an even higher sensitivity to attack by DNase I as compared with the active gene as a whole, pointing to highly localized alterations in chromatin structure (Wu, 1980; Stalder *et al.*, 1980; Cartwright *et al.*, 1982). These DNase I hypersensitive sites occur within ~200 bp of the start site, where they include the TATA box (Keene and Elgin, 1981; McGhee *et al.*, 1981; Cartwright *et al.*, 1982; Sweet *et al.*, 1982), and also in further upstream sequences (Chung *et al.*, 1983; Burch and Weintraub, 1983; Fritton *et al.*, 1983).

The ovalbumin gene in the chicken oviduct offers attractive features for studying the control of gene expression. It is transcribed at a high level in the oviduct of the laying hen, and the level of expression can be controlled by the administration or withdrawal of steroid hormones to immature chicks (Palmiter, 1975; Palmiter *et al.*, 1981). Probing the structure of the ovalbumin gene by digestion with DNase I has shown that the coding region and the flanking regions of the active gene in oviduct cells exhibit a generally elevated sensitivity to DNase I, as determined from the kinetics of rehybridization in solution, of DNA from DNase I digested nuclei (Garel and Axel, 1976; Lawson *et al.*, 1982; Stumpf *et al.*, 1983). This senstivity is higher in the 5'-flanking region than in the coding region of the gene (Bellard and Dretzen, unpublished results). When the analysis of the organization of ovalbumin gene chromatin was extended with the original blot hybridization technique of Wu *et al.* (1979) that first revealed hypersensitive sites in *Drosophila* chromatin, localized sites of DNase I hypersensitivity appeared to be absent from the 5'-flanking region (Bellard *et al.*, 1980). However, sub-optimal conditions for detecting hypersensitive sites were used in this study; the 5'-flanking region is very sensitive to DNase I, and a rather long probe was used.

A very different view of the organization of the 5'-flanking region of the active ovalbumin gene was obtained from the mapping of micrococcal nuclease (MNase) cleavage sites (Bellard *et al.*, 1982). Since MNase displays a strong sequence specificity (Dingwall *et al.*, 1981; Hörz and Altenburger, 1981), this analysis was done by comparison. The indirect end-labeling technique (Wu, 1980) was used to determine the position and intensity of MNase cleavage sites for a 2.5-kb segment of the ovalbumin gene 5'-flanking region, in oviduct where the gene is active, in erythrocytes where it is inactive, and in naked DNA. Over 20 of the >26 preferential cleavage sites that were identified in naked DNA, appeared not to be correlated with structural modifications specifically concerned with gene expression. For these, cleavages occurred with similar intensities at the corresponding sites in each of the three DNA samples. However, cleavages at the sites indicated N, P and (α) on the MNase cleavage map in Figure 1 (modified after Bellard *et al.*, 1982) were enhanced in oviduct nuclei; cleavages at Q, V and Y occurred only in oviduct nuclei, and cleavages at J and M were strongly suppressed in oviduct nuclei. Thus the analysis of cleavages produced by MNase provides clear evidence that the organization of the chromatin of the 5'-flanking region of the active gene is modified to yield different sensitivities to digestion by the enzyme at selected sites, compared with its organization in the inactive state in erythrocytes.

The MNase results prompted us to re-investigate the possible presence of DNase I hypersensitive sites in the ovalbumin

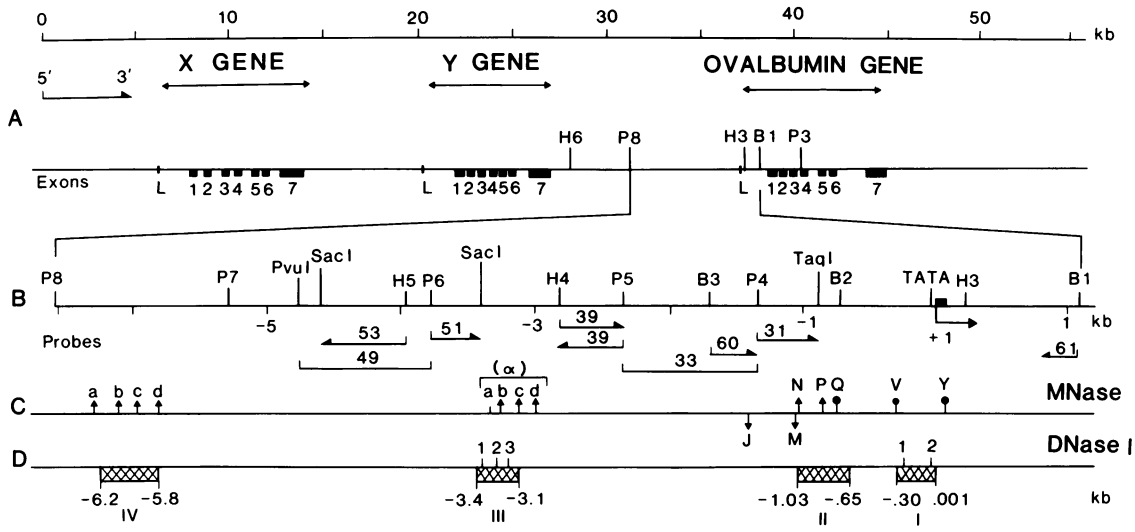

Fig. 1. **(A)** Genomic map of the ovalbumin gene family showing the X, Y and ovalbumin genes (Royal *et al.*, 1979; Heilig *et al.*, 1980). Selected *Hind*III (H), *Pst*I (P) and *Bgl*II (B) sites are shown as are the leader sequences (L) and the exons (solid bars). **(B)** A large scale map of the 5′-flanking region and part of the first intron of the ovalbumin gene from the *Pst*I8 (P8) site to the *Bgl*II1 (B1) site. The location of the *Bgl*II (B), *Pst*I (P) and *Hind*III (H) restriction sites used in the mapping of DNase I and MNase cleavage sites are indicated, as are the *Taq*I, *Pvu*I and *Sac*I sites utilized in making probes. The start site of transcription is located at position + 1 bp and is indicated by an arrow; TATA indicates the location of the TATA box. The various probes used in mapping are shown below the line. **(C)** A map of MNase cleavage sites showing sites that are enhanced in the active gene (arrows pointing upward), sites that are unique to the active gene (closed circles) and sites that are suppressed in the active gene (arrows pointing downwards). **(D)** A map showing the location of the DNase I hypersensitive regions I, II, III and IV as cross-hatched bars below the line and localized DNase I cleavage sites above the line.

5′-flanking region, but now utilizing the indirect end-labeling technique of Wu (1980) with short probes to increase the sensitivity of detection of such sites. We report here that four regions of DNase I hypersenstivity have been detected. In addition, the mapping of MNase cleavage sites was extended upstream from the 2.5-kb segment already examined (Bellard *et al.*, 1982), to include the entire 6.5-kb segment analyzed for the DNase I hypersensitivity mapping. Comparison of the cleavage patterns produced by the two enzymes shows that sites of enhanced or unique cleavages by MNase are closely associated with sites of DNase I hypersensitivity.

Results

The positions of DNase I and MNase cleavage sites were determined with the indirect end-labeling technique of Wu (1980). Briefly, isolated nuclei were subjected to mild digestion with the appropriate nuclease; the DNA was then purified, digested to completion with a restriction enzyme, electrophoresed, blotted and hybridized with appropriate radioactive probes, thereby labeling one end of a restriction fragment. A map of relevant restriction enzyme sites in a 6.5-kb segment of the 5′-flanking region and ~1 kb of the coding region of the ovalbumin gene is shown in Figure 1B, together with the probes which were used.

DNase I hypersensitive region I

A blot of a *Bgl*II redigest of the DNA from DNase I digested oviduct nuclei is shown in Figure 2, lanes 3–5. The DNA was hybridized with probe 61 (Figure 1B). The 1.85-kb *Bgl*II1-*Bgl*II2 (B1-B2) restriction fragment diminishes with increasing DNase I digestion of the nuclei (compare lanes 3 and 5). The band at 1.2–1.45 kb results from localized cleavages at a hypersensitive region, designated region I, which is centered ~150 bp upstream from the start site of the gene. Region I extends approximately from the start site of transcription at 1 bp to −300 bp as determined from measurements of the widths of sub-bands on a number of blots. It is indicated on the DNase I map in Figure 1D by a cross-hatched bar. Close inspection of the region I band in lane 3 reveals two faint sub-bands, 1 and 2. The cutting sites which generate these sub-bands are located approximately at −65 and −220 bp, respectively.

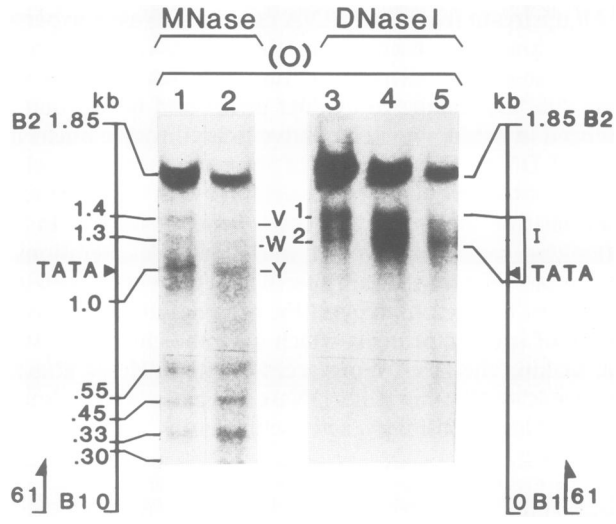

Fig. 2. MNase and DNAse I cleavage sites in oviduct nuclei (O) mapped in the 3′-5′ direction between the *Bgl*II1 and *Bgl*II2 (B1-B2) restriction sites. DNA from MNase and from DNase I digested nuclei was redigested with *Bgl*II, electrophoresed, blotted and hybridized with ^{32}P-labeled probe 61 (Figure 1B). The locations of MNase and DNase I cleavage sites are indicated on the maps of the B1-B2 fragment at the left and right of the figure. DNA from MNase digested nuclei (**lanes 1** and **2**) shows the B1-B2 fragment at 1.85 kb, and bands resulting from cleavages at MNase cleavage sites. DNA from DNase I treated nuclei (**lanes 3–5**) shows the B1-B2 fragment and the cleavage band between 1.45 and 1.2 kb that results from DNase I cleavage at DNase I hypersensitive region I. MNase concentrations, **lanes 1, 2**: 10, 23 units/ml. DNase I concentrations, **lanes 3–5**: 4, 6, 8 μg/ml.

Lanes 1 and 2 show DNA from MNase digested oviduct

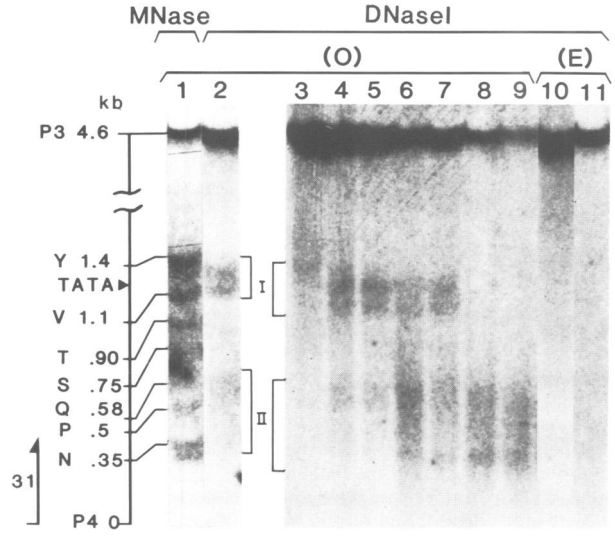

Fig. 3. MNase and DNase I cleavage sites in oviduct (O) and erythrocyte (E) nuclei mapped in the 5′-3′ direction between the *Pst*I4 and *Pst*I3 (P4-P3) restriction sites. DNA from MNase and DNase I digested nuclei was redigested with *Bgl*II, electrophoresed, blotted and hybridized with ^{32}P-labeled probe 31 (Figure 1B). The locations of MNase cleavage sites are shown on the map of the P3-P4 fragment at the left of the figure. DNA from MNase digested nuclei (**lane 1**) shows the P3-P4 fragment at 4.6 kb and bands resulting from MNase cleavages at specific cleavage sites. DNA from DNase I treated nuclei (**lane 2**) shows the cleavage bands resulting from cleavages in DNase I hypersensitive regions I and II. DNA from a DNase I digestion series of oviduct nuclei (**lanes 3–9**) shows the development of bands with increasing DNase I digestion. DNA from DNase I digested erythrocyte nuclei (**lanes 10** and **11**) shows the absence of specific cleavage sites. MNase concentration, **lane 1**: 20 units/ml. DNase I concentrations, **lanes 2–11:** 5.0, 0, 5.0, 5.8, 6.6, 7.5, 8.3, 9.0, 4.0, 5.0 μg/ml.

nuclei. The bands labeled V at 1.4 kb and Y at 1.0 kb from the *Bgl*II1 site are produced in oviduct nuclei, but not in erythrocyte nuclei or in naked DNA (Bellard *et al.*, 1982). The cleavage site V at −300 bp is within the DNase I hypersensitive region I; the site at Y is ~100 bp downstream from the edge of hypersensitive region I. The band at W and all of those of higher mobility than band Y are not specific to oviduct chromatin DNA (Bellard *et al.*, 1982).

Figure 3 shows blots of *Pst*I re-digested DNA hybridized with probe 31 (Figure 1B). The *Pst*I4-*Pst*I3 (P4-P3) restriction fragment is seen at 4.6 kb (note, the *Pst*I3 site is outside the restriction site map in Figure 1B, but is shown on the genomic map in Figure 1A). A DNase I digestion series of oviduct nuclei in lanes 3–9 shows the effect of increasing digestion on the development and disappearance of the sub-bands resulting from cleavages within hypersensitive region I. As the band centered at 1.2 kb disappears, it is replaced by bands of higher mobility between 0.3 and 0.6 kb from the *Pst*I4 site (see below). Lanes 10 and 11 show DNase I digested erythrocyte nuclei. The digestion products migrate as a uniform smear indicating the absence of hypersensitive sites. Analysis of DNA from oviduct nuclei digested with MNase (lane 1) and DNase I (lane 2) shows that the MNase cleavage band V is at the edge of DNase I hypersensitive region I and band Y is adjacent to the edge. The MNase bands T and S occur in erythrocyte nuclei and in naked DNA (Bellard *et al.*, 1982), with the intensities seen here for the oviduct nuclei.

DNase I hypersensitive region II

A DNase I hypersensitive region designated region II was mapped between −0.65 and −1.03 kb, in *Pst*I, *Bgl*II and

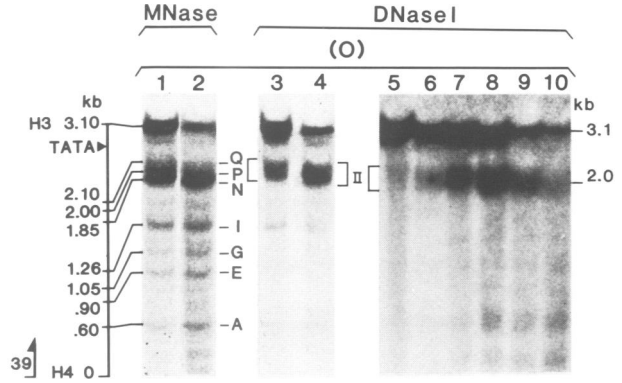

Fig. 4. MNase and DNase I cleavage sites in oviduct (O) nuclei mapped in the 5′-3′ direction between the *Hind*III4-*Hind*III3 (H4-H3) restriction sites. DNA from MNase and from DNase I digested nuclei was redigested with *Hind*III, electrophoresed, blotted and hybridized with ^{32}P-labeled probe 39 (Figure 1B). The locations of the MNase cleavage sites are indicated on the map of the H4-H3 restriction fragment at the left of the figure. DNA from MNase digested nuclei (**lanes 1** and **2**) shows the H4-H3 restriction fragment at 3.10 kb, and bands resulting from cleavages at specific MNase cleavage sites. DNA from a DNase I digested nuclei (**lanes 3** and **4**) shows the broad cleavage band resulting from multiple cleavages within hypersensitive region II. DNA from DNase I digestion series of oviduct nuclei (**lanes 5–10**) shows the development of the cleavage band with increasing DNase I digestion. MNase concentrations, **lanes 1** and **2**: 10, 23 units/ml. DNase I concentrations, **lanes 3–10**: 4.0, 6.1, 3.3, 5.0, 5.8, 6.6, 7.5, 8.3 μg/ml.

*Hind*III redigests. The bands which were noted above in *Pst*I redigests between 0.3 and 0.6 kb from the *Pst*I4 site result from cleavages by DNase I in region II (Figure 3, lanes 3–9). There are also three MNase cleavage sites within region II that are detected in *Pst*I redigests. The strong bands at N and P (Figure 3, lane 1) result from MNase cleavages that are enhanced in oviduct nuclei, and the weaker band at Q results from cleavages at a site that is unique to the oviduct (Bellard *et al.*, 1982; see Figure 1C).

DNase I cleavages in region II were also revealed in *Hind*III redigests hybridized with probe 39 (Figure 1B). Figure 4 (lanes 5–10) shows a DNase I digestion series. The *Hind*III4-*Hind*III3 (H4-H3) restriction fragment is at 3.1 kb and the sub-bands resulting from cleavages in region II are centered at 2.0 kb from the *Hind*III4 site. A close comparison of the DNase I cleavages with those produced by MNase is shown in lanes 1–4. Lanes 1 and 2 show MNase digestion of oviduct nuclei with the products of cleavages produced by MNase at the Q, P and N sites, all located within the same region of the H4-H3 fragment as the products of DNase I cleavages in hypersensitive region II (lanes 3 and 4). N is the strongest band and is at the edge of DNase I hypersensitive region II. The cleavage sites at A, E, G and I (lanes 1 and 2) are present in chromatin DNA of the erythrocyte and in naked DNA, with intensities similar to that seen in chromatin DNA of oviduct cells (Bellard *et al.*, 1982).

DNase I hypersensitive region II was also mapped in *Bgl*II redigests hybridized with probe 60 (Figure 1B). Figure 5 (lanes 5–10) shows DNA from oviduct nuclei digested with DNase I. The upper boundary of the products of digestion in region II is the *Bgl*II2-*Bgl*II3 (B2-B3) restriction fragment at 1 kb, as expected since the *Bgl*II2 site is within region II. From there, the cleavage products extend downward in the gel to about the 0.7-kb position, terminating in a distinct band. Specific cleavage sites are not observed in DNase I digested erythrocyte nuclei (lanes 11 and 12). A comparison of

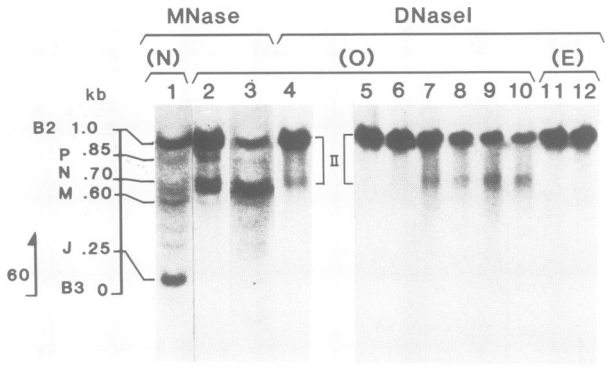

Fig. 5. MNase and DNase I cleavage sites mapped in the 5′-3′ direction between the *Bgl*II3-*Bgl*II2 (B3-B2) restriction sites. DNA from MNase digested naked DNA (N) and oviduct (O) nuclei, and DNA from DNase I digested oviduct and erythrocyte (E) nuclei was redigested with *Bgl*II, electrophoresed, blotted and hybridized with ^{32}P-labeled probe 60 (Figure 1B). The locations of the MNase cleavage sites are indicated on the map of the B3-B2 fragment at the left of the figure. Naked DNA digested with MNase (**lane 1**) and DNA from oviduct nuclei digested with MNase (**lanes 2, 3**) shows the B3-B2 fragment at 1.0 kb and the bands resulting from cleavages at specific MNase cleavage sites. DNA from DNase I digested oviduct nuclei (**lane 4**) shows a broad cleavage band resulting from cleavages at DNase I hypersensitive region II. DNA from a DNase I digestion series of oviduct nuclei (**lanes 5–10**) shows the development of the cleavage band with increasing DNase I digestion. DNA from DNase I digested erythrocytes nuclei (**lanes 11** and **12**) shows the absence of specific cleavage sites. MNase concentrations, **lanes 1–3**: 6, 10, 50 units/ml. DNase I concentrations, **lanes 4–12**: 6.0, 4.8, 6.4, 8.0, 9.6, 11.2, 11.8, 8.1, 12.8 μg/ml.

cleavages produced by MNase and by DNase I is shown in lanes 1–4. Weak cleavages at the P and N sites and strong cleavages at the J site are seen after MNase digestion of naked DNA (lane 1); they should be compared with the enhanced cleavages at P and N and the suppressed cleavages at J and M in the oviduct samples (lanes 2 and 3) (see Bellard *et al.*, 1982 and Figure 1C). Cleavages at P and at N are within region II and at the edge of region II respectively, as demonstrated by comparison with the DNase I digested oviduct nuclei (lane 4). Cleavages at site Q are not seen here beause the products are included within the band formed by the *Bgl*II2-*Bgl*II3 (B2-B3) restriction fragment.

DNase hypersensitive region III

A region of DNase I hypersensitivity was mapped between −3.1 and −3.4 kb in *Pst*I redigests hybridized with probe 51 and with probe 39 (Figure 1B). Figure 6 shows *Pst*I redigested DNA hybridized with probe 51. The *Pst*I5-*Pst*I6 (P5-P6) restriction fragment migrates at 1.45 kb. The products of cleavages in hypersensitive region III (lanes 9–11) extend ~0.7 to 0.4 kb from the *Pst*I6 site. Three preferential cutting sites (1, 2 and 3) are detected within region III. As might be expected, the fragments produced by cleavages at site 3 which is most distal to the *Pst*I6 site labeled by probe 51, diminish with increasing digestion and the shorter fragments produced at sites 1 and 2 accumulate. The band at 0.3 kb from the *Pst*I6 site was not reproducibly observed.

There are three strong MNase cleavage sites in oviduct nuclei in the segment of DNA corresponding to DNase I hypersensitive region III. Cleavages at these sites produce the bands designated a, b and c (Figure 6, lanes 6–8). (In a preliminary account of these sites they were called the α sites, Bellard *et al.*, 1982.) There is also a MNase cleavage site

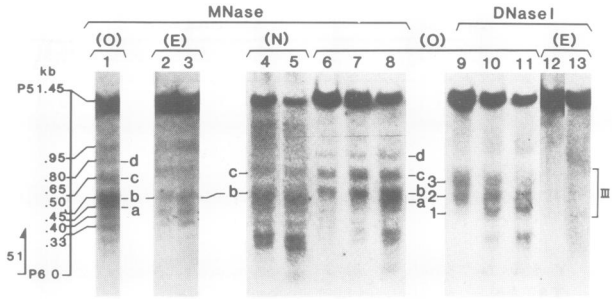

Fig. 6. MNase and DNase I cleavage sites mapped in the 5′-3′ direction between the *Pst*I6-*Pst*I5 (P6-P5) restriciton sites. DNA from MNase and from DNase I digestion of oviduct (O) and erythrocyte (E) nuclei and from MNase digestion of naked (N) DNA was redigested with *Pst*I, electrophoresed, blotted and hybridized with ^{32}P-labeled probe 51 (Figure 1B). The locations of the MNase cleavage sites are indicated on the map of the P6-P5 fragment at the left of the figure. The P6-P5 fragment is at 1.45 kb in all lanes. DNA from MNase digested oviduct nuclei (**lanes 1, 6–8**) and erythrocyte nuclei (**lanes 2** and **3**), and from MNase digested naked DNA (**lanes 4** and **5**) shows bands resulting from cleavages by MNase at specific MNase cleavage sites. DNA from DNase I digested oviduct nuclei shows bands 1–3 resulting from cleavages at DNase I hypersensitive region III (**lanes 9–11**). DNA from DNase I digested erythrocyte nuclei (**lanes 12** and **13**) shows no specific cleavage bands. MNase concentrations, **lanes 1–8**: 60, 13, 55, 6, 10, 6.6, 10, 35 units/ml. DNase I concentrations, **lanes 9–13**: 4, 6, 8, 4, 50 μg/ml.

which is close to, but outside of region III; the cleavages here produce the band designated d (lanes 6–8). MNase also cleaves naked DNA at sites which may correspond to sites b, c and d (lanes 4 and 5), but relative to these cleavage sites they are weaker in naked DNA than in oviduct nuclei. The presence of a discrete cleavage site at 'a' could not be determined in naked DNA. A cleavage site at b is present in erythrocyte nuclei, but is relatively weaker than in oviduct nuclei (compare lanes 2 and 3 with lane 1). Cleavage sites at a, c and d may be absent from erythrocyte nuclei. Electrophoretic bands that would result from cleavages at these sites are not evident in DNA from these nuclei. A band having a mobility intermediate between that of the bands designated c and d does, however, occur in erythrocytes. This band is unique to the erythrocyte and has not been investigated further.

Cleavages within region III were also detected in *Pst*I redigests hybridized with probe 39 (Figure 7). A DNase I digestion series of oviduct nuclei (lanes 7–11) shows the *Pst*I5-*Pst*I6 (P5-P6) restriction fragment at 1.45 kb, and subbands resulting from cleavages in region III in the region between 0.8 kb and 1.0 kb from the *Pst*I5 site. Only the least digested material (lane 7) shows the cleavage sub-bands 2 and 3 which are clearly seen in blots hybridized with probe 51 (Figure 6). (Sub-band 1 is not seen in Figure 7, because it is the most distal from the *Pst*I5 site). There are no evident cleavage bands in DNA from DNase I digested erythrocyte nuclei (Figure 7, lanes 12 and 13). Comparison of MNase-digested naked DNA (lanes 1 and 2) with DNA from oviduct nuclei (lanes 3–5), shows that cleavage sites b, c and d which are strong in oviduct nuclei are much weaker in naked DNA. The MNase site c located at the 3′ edge of region III appears to be the strongest of these MNase cleavage sites (Figures 6 and 7). Since in Figure 6 any cleavages at site b would diminish the intensity of band c, more cleavages must occur at c than at b to yield the equal intensities of bands b and c seen in these blots.

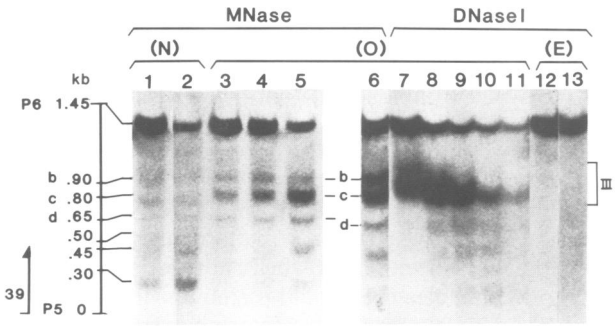

Fig. 7. MNase and DNase I cleavage sites mapped in the 3′-5′ direction between the *Pst*I5-*Pst*I6 (P5-P6) restriction sites. MNase digested naked (N) DNA, DNA from MNase digested oviduct (O) nuclei, and DNA from DNase I digested oviduct and erythrocyte (E) nuclei was redigested with *Pst*I, electrophoresed, blotted and hybridized with ^{32}P-labeled probe 39 (Figure 1B). The locations of MNase cleavage sites are indicated on the map of the P5-P6 fragment to the left of the figure. The P5-P6 fragment is at 1.45 kb in all lanes. DNA from MNase digested oviduct nuclei (**lanes 3–6**) and from MNase digested naked DNA (**lanes 1** and **2**) shows bands resulting from MNase cleavages at specific MNase cleavage sites. DNA from a DNase I digestion series of oviduct nuclei (**lanes 7–11**) shows cleavage bands resulting from cleavages in DNase I hypersensitive region III. DNA from DNase I digested erythrocyte nuclei (**lanes 11** and **12**) shows no specific cleavage bands. MNase concentrations, **lanes 1–6**: 0.8, 8, 6.5, 10, 35, 20 units/ml. DNase I concentrations, **lanes 7–13**: 5, 7, 9, 11, 13, 4, 50 μg/ml.

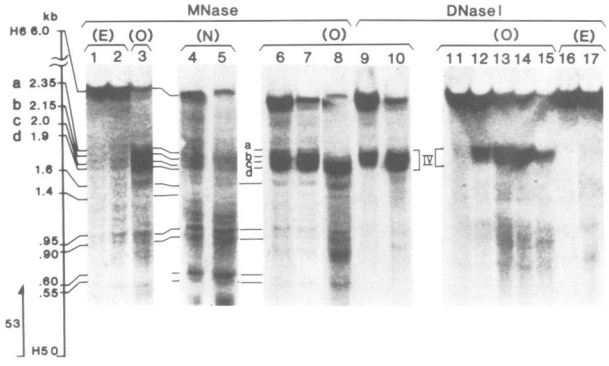

Fig. 8. MNase and DNase I cleavage sites mapped in the 3′-5′ direction between the *Hind*III5-*Hind*III6 (H5-H6) restriction sites. DNA from MNase digested oviduct (O) and erythrocyte (E) nuclei, MNase digested naked DNA (N), and DNA from DNase I digested oviduct and erythrocyte nuclei was redigested with *Hind*III, electrophoresed, blotted and hybridized with ^{32}P labeled probe 53. The locations of MNase cleavage sites are indicated on the map of the H5-H6 restriction fragment at the left of the figure. The H5-H6 restriction fragment is at 6 kb. DNA from MNase digested oviduct (**lanes 3, 6–8**) and erythrocyte nuclei (**lanes 1** and **2**) and from MNase digested naked DNA (**lanes 4** and **5**) shows bands which result from MNase cleavages at specific sites. DNA from DNase I digested oviduct nuclei (**lanes 9–15**) shows the cleavage bands resulting from cleavages in the DNase I hypersensitive region IV. A digestion series showing the development of region IV cleavage bands with increasing DNase I concentrations is shown in **lanes 11–15**. DNA from DNase I digested erythrocyte nuclei (**lanes 16** and **17**) shows several faint cleavage bands. MNase concentrations, **lanes 1–8**: 100, 175, 60, 0.8, 6, 6.5, 10, 50 units/ml. DNase I concentrations, **lanes 9–17**: 5.0, 7.5, 0, 6.4, 8.0, 9.6, 11.2, 4.8, 12.8 μg/ml.

DNase I hypersensitive region IV

A DNase I hypersensitive region was observed between −5.8 and −6.2 kb in *Hind*III redigested oviduct chromatin DNA hybridized with probe 53 (Figure 1B). A DNase I digestion series is shown in Figure 8, lanes 11–15. The *Hind*III5-*Hind*III6 (H5-H6) restriction fragment is seen at 6 kb, and sub-bands produced by preferential DNase I cleavages within

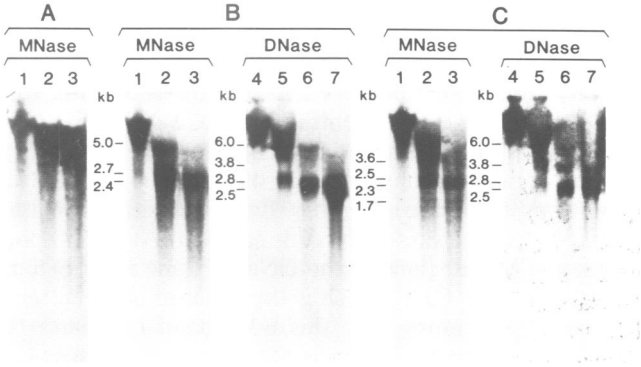

Fig. 9. Specific cleavage fragments produced by MNase and by DNase I digestion of oviduct nuclei. DNA from digested nuclei was electrophoresed without redigestion with restriction enzyme, blotted and hybridized with ^{32}P-labeled probes. (**A**) Hybridization with a probe for the β-globin gene. (**B**) DNA from MNase (**lanes 1–3**) and DNase I (**lanes 4–7**) digested nuclei hybridized with probe 49 (Figure 1B). Cleavage bands are seen in each at ~2.5 kb at a high level of digestion (**lanes 3** and **7**) and also at ~6 kb at lower levels of digestion (**lanes 2, 5** and **6**). (**C**) DNA from MNase (**lanes 1–3**) and DNase I (**lanes 4–7**) digested nuclei hybridized with probe 33 (Figure 1B). A strong cleavage band is seen at ~2.4 kb in each (**lanes 3** and **7**). Cleavage bands are also seen at ~6 and 3.6 kb (**lanes 2, 5** and **6**) at lower levels of digestion. MNase concentrations, **lanes 1–3**: 20, 60, 90 units/ml. DNase I concentrations, **lanes 4–7**: 4.8, 6.4, 8.0, 9.6 μg/ml.

region IV at 2 kb. DNA from DNase I digested erythrocyte nuclei (lanes 16 and 17) shows a faint banding pattern, but no strong sites of hypersensitivity.

DNA from MNase digested oviduct nuclei displays a series of sharp bands in region IV in the original autoradiographs, but these are not evident in the reduced photographs reproduced in Figure 8. The bands designated b, c and d are produced by cleavages at sites included within region IV, whereas band a is produced by cleavages at a site which is located ~200 bp upstream from region IV. Correspondingly sharp cleavage patterns were not evident in the DNase I treated nuclei, but the band resulting from cleavages in region IV is quite wide and there is a shift to smaller sized fragments with increasing concentration of DNase I, which suggests the presence of more than one cleavage site (see lanes 9 and 10).

Comparison of MNase digests of oviduct nuclei with those of naked DNA (Figure 8, lanes 6–8 *versus* 4 and 5), and of erythrocyte nuclei (Figure 8, lane 3 *versus* 1 and 2) shows that sites a, b, c and d are present in all three but, relative to other cleavage sites, they are much weaker in erythrocyte nuclei and naked DNA than in oviduct nuclei.

Blots of DNA not redigested with restriction enzymes

The presence of DNase I hypersensitive cleavage sites in oviduct chromatin at the four regions described above leads to the prediction that mild DNase I digestion should release discrete chromatin fragments. That is, DNase I should produce fragments that span the segments between regions I and II, between II and III, between III and IV, etc. Blots of DNA from DNase I digests that had not been redigested with restriction enzyme were prepared and similar blots were made with non-redigested DNA from MNase digested nuclei. These blots were hybridized with probe 33, which is located between regions II and III, and with probe 49, located between regions III and IV (Figure 1B). The results are shown in Figure 9. In 9A, lanes 1–3, DNA from MNase digested oviduct nuclei was hybridized with a β-globin gene as a control; there is no

indication of specific cleavage sites. In 9B the same blot is shown (lanes 1–3) together with a blot of DNA from DNase I digested oviduct nuclei (lanes 4–7), both having been hybridized with probe 49. At the highest MNase (lane 3) and DNase I (lane 7) concentrations, cleavage bands are seen at ~2.5 kb. This is just the distance between regions III and IV, the region containing probe 49. This observation confirms that there are no other highly sensitive MNase or DNase I cleavage sites within this segment. At lower concentrations of MNase (lane 2) and DNase I (lane 5 and 6) a band appears at ~5–6 kb, which is the distance between regions I-II to IV suggesting that this 6-kb band originates from cleavages at these regions, prior to the more complete digestion seen in lanes 3 and 7.

The same blots were hybridized with probe 33 as shown in Figure 9C. At the highest MNase (lane 3) and DNase I (lane 7) concentrations, a strong band is seen at ~2.4 kb which is the distance between the centers of regions II and III, the segment containing probe 33. At lower MNase (lane 2) and DNase I (lanes 5 and 6) concentrations, two additional bands appear at ~3.6 and 6 kb, sizes which are consistent with their production by cleavages in regions I and III, and in regions I-II and IV, respectively, prior to the more complete digestions seen in lanes 3 and 7.

In control experiments (results not shown) DNA from oviduct nuclei which had been incubated in the absence of DNase I or MNase displayed no specific cleavage fragments. DNA from DNase I or MNase digested erythrocyte nuclei migrates as a uniform smear.

Discussion

In the 6.5-kb segment of the ovalbumin gene 5′-flanking region studied here, there are four regions of DNase I hypersensitivity centered at 0.15, 0.80, 3.2 and 6.0 kb upstream from the mRNA cap site, respectively. The hypersensitivity is correlated with gene activity because it is observed in oviduct cells of the laying hen, where the gene is expressed, but is absent from erythrocytes where it is not expressed. The MNase cleavage sites that are enhanced in, or are unique to the active gene (Bellard *et al.*, 1982, and the present study) are closely associated with the DNase I hypersensitive regions. They are located within them, or within 200 bp of their boundaries. Confirmation that hypersensitivity to each enzyme is restricted to the same regions comes from the observations that both MNase and DNase I digestion releases large fragments, apparently identical, which span the flanking region between hypersensitive regions I-II and III, I-II and IV, and III and IV.

A simple way to explain the association of hypersensitivities to MNase and to DNase I is that DNA at hypersensitive sites is free of protein and is thus accessible to each enzyme. Evidence consistent with this idea has been obtained for a 200-bp segment of the 5′-flanking region adjacent to the start site of the chicken β-globin gene which exhibits hypersensitivity to several endonucleases including DNase I (McGhee *et al.*, 1981). Up to 60% of the DNA from this segment migrates with the electrophoretic mobility of naked DNA, when a restriction digest of chromatin is electrophoresed. We have not tested directly for the presence of naked DNA in the hypersensitive regions of the ovalbumin gene but, even if some is present there, it cannot be the only reason for the close association of hypersensitivities towards DNase I and MNase. Because of its high sequence specificity, MNase produces a characteristic cleavage pattern in naked DNA (Dingwall *et al.*, 1981; Hörz and Altenburger, 1981). That cleavage pattern is altered in the ovalbumin gene at each of the hypersensitive regions (Bellard *et al.*, 1982; and the present study), which is contrary to the results expected if the DNA there were simply naked.

It has been proposed (Bellard *et al.*, 1982) that in the 5′-flanking region of the active ovalbumin gene, proteins bind at the MNase cleavage sites that are unique to the active gene, and de-stabilize the DNA helix at those sites. High sensitivity to MNase cleavage would thereby be induced because MNase has a strong preference for single-strand DNA (von Hippel and Felsenfeld, 1964). Indirect support for this proposal comes from the finding that single-strand DNA may, in fact, occur in DNase I hypersensitive regions in the active chicken β-globin gene, as detected by cleavage with single-strand-specific nucleases (Larsen and Weintraub, 1982; Weintraub, 1983) and by attack with the single-strand-specific reagent bromoacetaldehyde (Kohwi-Shigematsu *et al.*, 1983).

Helix destabilization by proteins cannot explain the hypersensitivity to DNase I because DNase I has a preference for double-stranded DNA (Douvos and Price, 1975). Conceivably, however, DNase I hypersensitivity might nevertheless be the consequence of specific protein binding if, in a way not understood, the proteins induce changes in the DNA making it a preferred substrate for DNase I. That appears to be the case with gyrase binding to DNA (Fisher *et al.*, 1981) and with Sp1 transcription factor binding to SV40 DNA (Dynan and Tjian, 1983). It is also conceivable that binding of specific proteins at hypersensitive sites alters the structure of chromatin to give an open arrangement that is highly accessible to attack by DNase I.

DNase I hypersensitive sites found close to the start sites of transcription have been correlated indirectly with promoter functions localized in those regions (for reviews, see Elgin, 1981; Cartwright *et al.*, 1982). A detailed correlation has been obtained for the herpes virus thymidine kinase gene in which the promoter region extends to −105 bp from the start site (McKnight and Kingsbury, 1982). Hypersensitivity to DNase I and to several restriction enzymes extends through the whole of the promoter region (Sweet *et al.*, 1982). In the ovalbumin gene, DNase I hypersensitivity adjacent to the start site also appears to be correlated with promoter functions. Hypersensitive region I, which extends approximately from the cap site to −300 bp, includes the TATA sequence at −26 to −31 bp and the CAAT sequence at −69 to −77 bp (Benoist *et al.*, 1980). In addition, hypersensitivity in region I seems to be correlated with the regulation of the gene by steroid hormones. A binding site for the progesterone receptor is located within hypersensitive region I (Mulvihill *et al.*, 1982; Compton *et al.*, 1983). Furthermore, a direct stimulatory effect of progesterone and estrogen on transcription mediated by the ovalbumin gene 5′-flanking region included in region I has been shown using chimeric recombinants which were introduced into primary cultures of oviduct cells (Dean *et al.*, 1983; Knoll *et al.*, 1983; Chambon *et al.*, 1984; M.P. Gaub and A. Dierich, personal communication).

Functional correlates are more problematical for the DNase I hypersensitive regions upstream from region I. There is a precedent from other genes for transcriptional control elements which are located at some distance from the start site. In the *Sgs4* glue gene of *Drosophila*, expression is

dependent on the presence of a region between −330 and −550 bp which is hypersensitive to DNase I (Shermoen and Beckendorf, 1982; McGinnis *et al.*, 1983a, 1983b). In the SV40 minichromosome, the 72-bp repeat enhancer sequence which is hypersensitive to DNase I (Saragosti *et al.*, 1982; Fromm and Berg, 1983; Jongstra *et al.*, 1984) is normally located at coordinates −107 to −270 bp, but it shows a stimulatory effect on transcription even when it is removed as far as 3.5 kb upstream in artificial constructs (for references, see Moreau *et al.*, 1981; Wasylyk *et al.*, 1983) and it induces DNase I hypersensitivity at its insertion locations (Jongstra *et al.*, 1984). Enhancer elements which are located downstream from the start site of transcription have been found in the immunoglobulin μ heavy chain genes (Gillies *et al.*, 1983; Banerji *et al.*, 1983) and in the κ light chain genes (Queen and Baltimore, 1983; Picard and Schaffner, 1984). In both cases, the location of the enhancer element is associated with a region of DNase I hypersensitivity (Mills *et al.*, 1983; Parslow and Granner, 1982; Weischet *et al.*, 1982; Chung *et al.*, 1983; Picard and Schaffner, 1984). We speculate that one or more of the upstream hypersensitive regions in the ovalbumin gene may be the site of an enhancer element, perhaps controlled by steroid hormone receptor binding. We note that the *Pst*I4-*Bgl*II2 segment of the ovalbumin gene flanking region, which includes hypersensitive region II, has an elevated affinity for binding the progesterone receptor. It contains three sequences which show strong homology with the consensus sequence suggested for the binding of the hormone receptor and these are located in the segment which is encompassed by hypersensitive region II (Mulvihill *et al.*, 1982). Little is known about hypersensitive regions III and IV, for which sequencing and steroid hormone receptor binding studies are lacking. We note that region IV is far upstream from the start site, at −6.0 kb, and conceivably it could be associated with the functioning of the adjacent Y gene (Figure 1A) rather than with that of the ovalbumin gene. Region IV is nearly equidistant from the termination site of the Y gene and start site of the ovalbumin gene.

The DNA of the 5′-flanking region of the active ovalbumin gene appears to be preferentially associated with the nuclear matrix of oviduct cells (Robinson *et al.*, 1983; Ciejek *et al.*, 1983). Since it has been suggested that DNA may be attached to the nuclear matrix through matrix-bound hormone receptors (Robinson *et al.*, 1983; Butthyan *et al.*, 1983), it would be of interest to see whether the locations of the present nuclease hypersensitive regions correspond to attachment sites. Such comparisons require a precise mapping of the locations of the putative attachment sites which is not yet available (Robinson *et al.*, 1983).

We are currently testing the possible effects of the four nuclease hypersensitive regions on ovalbumin gene transcription and determining whether such effects might be dependent upon steroid hormones. These experiments should shed further light on the possible function of these 5′-flanking region segments which exhibit drastically altered chromatin structure when the ovalbumin gene is actively transcribed.

Materials and methods

Nuclei were isolated from oviducts and erythrocytes of laying hens and digested with MNase as described previously (Bellard *et al.*, 1982). For DNase I digestion, nuclei were washed twice in DNase I digestion buffer (85 mM KCl, 1 mM $CaCl_2$, 5 mM PIPES buffer pH 7.0, 5% sucrose), resuspended at a concentration of 1 mg DNA/ml, and digested at 20°C for 20 min. Enzyme concentrations are indicated in the figure legends. Digestions were terminated by addition of an equal volume of 'stop' solution (150 mM NaCl, 15 mM EDTA, 50 mM Tris-HCl pH 7.5, 0.3% SDS). The DNA was purified by digestion with RNase and proteinase K, followed by phenol-chloroform extractions. Naked DNA was prepared from erythrocyte nuclei as described (Bellard *et al.*, 1980) and digested with MNase and DNase I essentially as for nuclei, but for shorter times and with generally lower concentrations of enzyme. Restriction enzymes *Bgl*II, *Pst*I and *Hind*III were used according to the instructions of the suppliers.

Electrophoresis in 1.8% agarose gels, transfer to DBM-paper, the use of restriction digests of adenovirus-2 and SV40 DNAs as size markers, preparation and labeling of probes and hybridization with the DNA on blots were done as described (Dretzen *et al.*, 1981; Bellard *et al.*, 1982).

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