Chromatin Structure, Transcription, and Methylation of the Prolactin Gene Domain in Pituitary Tumors of Fischer 344 Rats*

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In order to study prolactin gene transcription and chromatin structure in a relatively homogeneous population of prolactin-producing cells, we induced lactotroph proliferation (pituitary tumor formation) in Fischer 344 rats by chronic diethylstilbestrol treatment. We show that the prolactin gene is highly sensitive to digestion by DNase I in the chromatin of these pituitary tumors but not in liver. Furthermore, sequences immediately flanking the prolactin gene exhibit a similar high DNase I sensitivity. This contrasts with previous reports that the coding region of some genes is highly sensitive to DNase I digestion and that adjacent noncoding regions exhibit an intermediate sensitivity. This high level of DNase I sensitivity in the flanking regions was not due to transcription; we did not detect RNA transcripts homologous to any unique DNA sequence within 12 kilobase pairs upstream or downstream of the prolactin gene. We have identified two DNase I-hypersensitive sites 5' to the prolactin gene in pituitary tumors but none in liver. Also, the coding region, but not adjacent noncoding regions, of the prolactin gene domain is hypomethylated at MspI/HpaII and HhaI restriction enzyme sites in pituitary tumors. The prolactin gene domain is methylated at these sites in liver.

Estrogen increases prolactin mRNA levels (1, 2) and the rate of transcription of the prolactin gene (3, 4) in rat anterior pituitaries. It has been hypothesized that estrogen modulates gene activity through a specific nuclear localization of the estrogen receptor. Interaction of the estrogen-receptor complex with a specific DNA sequence has been suggested as a possible mechanism; but as hormone-inducible genes are transcribed only in one or a few tissues out of the many with functional estrogen receptors, other factors also must be involved. It has been suggested that certain "developmentally significant" events must occur for hormonal controls to function (5).

Several structural features of chromatin have been associated with transcriptionally active genes. These include a general DNase I sensitivity of the chromatin gene domain (6, 7), DNase I-hypersensitive sites located near regulatory regions (8), hypomethylation of cytosine residues (9, 10), and binding of high mobility group proteins 14 and 17 to transcribed gene regions (11). Reports from two research groups have indicated that coding regions of the α- and β-globin genes (12, 13) and the ovalbumin gene (14) of the chicken are highly DNase I-sensitive. Adjacent noncoding regions (or previously transcribed embryonic genes) were reported to display an intermediate sensitivity to DNase I digestion. More recently, it has been reported that coding and immediately adjacent nontranscribed DNA sequences exhibit the same high level of sensitivity to DNase I digestion (15-17).

Knowledge of the chromatin structure of the prolactin gene will be important in understanding how this gene acquires the capability to respond to estrogen and the specific mechanisms of this response. In this report, we examine the tissue specificity of the DNase I sensitivity of the prolactin gene chromatin in diethylstilbestrol-treated rats of the Fischer 344 strain (18). We use limited nicking with DNase I coupled with filter blot hybridization to examine the relative DNase I sensitivity of the coding and noncoding regions of 35 kilobase pairs of the prolactin gene domain in pituitary tumors and liver. We also identify DNase I-hypersensitive sites near the prolactin gene, quantitate transcription from the prolactin gene domain, and examine the tissue-specific methylation pattern of the prolactin gene.

MATERIALS AND METHODS

Animals—F3441 male rats were obtained from Harlan-Sprague Dawley, Madison, WI. These rats were chronically treated with diethylstilbestrol by implanting 1-cm sections of silastic tubing containing 5 mg of DES under the skin of 3-week-old rats (19). The rats were then maintained on a 12-h light/12-h dark cycle with continuous access to rat chow and water.

Source of Cloned DNA—Approximately 37 kb of cloned prolactin genomic DNA was provided by Dr. Brad Thompson, National Institutes of Health (20). Serum albumin cDNA was provided by Dr. Richard Hanson, Case Western University (21).

Preparation and Digestion of Nuclei—F344 rats, treated with DES for 3 months (unless specified differently in the text), were anesthetized and decapitated. Pituitary tumors were removed and immediately placed in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.5, 20 mM KCl, 5 mM MgCl2, 0.5 mM sucrose, 0.15 mM spermine, and 0.5 mM spermidine). All subsequent steps were done at 4 °C. The pituitaries were homogenized by 10 strokes, at medium speed, of a motor-driven Teflon-glass homogenizer. The homogenate was layered onto a 0.85 M sucrose cushion in homogenization buffer and centrifuged 5 min at 5000 rpm in an HB-4 rotor (Sorvall). The pelleted nuclei were resuspended in homogenization buffer and again pelleted through a 0.85 M sucrose cushion as above. Liver nuclei were prepared as above except they were homogenized again before pelleting through the second sucrose cushion.

Nuclei were resuspended in DNase I digestion buffer (6) (10 mM Tris-HCl, pH 7.5, 2.5 mM MgCl2, 0.25 mM sucrose) to a DNA concentration of 1-2 mg/ml. Various concentrations of DNase I (Worthing-
tos, DPPF) were added to identical aliquots of nuclei, and the mixture was incubated at 37°C for 10 min. Digestion was stopped by adding EDTA to a final concentration of 10 mM and mixing vigorously.

**Restriction Endonuclease Cleavage of DNA, Transfer to Nickelcelelo, and Hybridization**—DNA was purified from the nuclei as previously described (22). Aliquots of purified DNA (30 µg) were digested to completion with an excess of restriction endonucleasr to a modification site recommended by the manufacturers (Bethesda Research Laboratories or New England Biolabs). After digestion, the DNA was fractionated by agarose gel electrophoresis in TEA buffer (40 mM Tris-HCl, 55 mM sodium acetate, 20 mM EDTA, pH 8.5). DNA was transferred from the gel to nitrocellulose filters (Schleicher & Schuell, BA85) as described (23). After overnight transfer, the filters were blotted dry and baked 2 h at 80°C. All hybridization and wash steps were done at 68°C (unless specified) as described previously (24). Briefly, the filters were washed 30 min with 3 X SSC (1 X SSC is 0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0). Next the filters were treated 3 h with 3 X SSC and 0.1% polyvinylpyrrolidone, 0.1% Ficoll, and 0.1% bovine serum albumin (modification of the procedure of Denhardt (25)). This solution was replaced with a similar solution that contained, in addition, 20 mM NaHPO₄, 0.1% SDS, and 50 µg/ml of denatured herring sperm DNA. The incubation was continued for 2 h; this solution was then replaced with a minimal volume of identical solution plus 1 X 10⁶ cpm/ml of 3²P-labeled DNA. Hybridization was continued 20–36 h. The nitrocellulose filters were washed three times with hybridization solution minus the labeled probe at room temperature and 30 min at 68°C. A final 30 min wash was with 0.1 X SSC and 0.1% SDS at 65°C. Filters were rinsed at room temperature with 3 X SSC and blotted dry. The dried filters were autoradiographed by exposing them to Kodak XAR-5 film for approximately 26 h to −80°C with a DuPont Cronex Lightning Plus intensifying screen.

**Preparation and Labeling of Hybridization Probes**—Fragments of DNA spanning the prolactin gene domain have been subcloned into the plasmid pBR322. Plasmid DNA containing different prolactin gene fragments was isolated (26), and the prolactin DNA was excised from the plasmid by restriction endonuclease cleavage. Prolactin DNA fragments were purified from plasmid DNA by preparative agarose gel electrophoresis, transferred to nitrocellulose filters (Schleicher & Schuell, BA85) as described (23). After overnight transfer, the filters were blotted dry and baked 2 h at 80°C. All hybridization and wash steps were done at 68°C (unless specified) as described previously (24). Briefly, the filters were treated 3 h with 3 X SSC and 0.1% polyvinylpyrrolidone, 0.1% Ficoll, and 0.1% BSA. Hybridizations were done in fresh prehybridization buffer plus 1 X 10⁶ cpm/ml of nick-translated DNA at 42°C for 36 h. The filters were washed with 3 X SSC, 0.1% SDS three times at room temperature and once for 30 min at 50°C. A final stringent wash was with 0.1 X SSC, 0.1% SDS at 50°C for 30 min. The filters were rinsed with 5 X SSC at room temperature, air-dried, and exposed to Kodak XAR-5 film for 3–24 h at −80°C with a DuPont Cronex Lightning Pius intensifying screen.

**Quantification of RNA**—The amount of prolactin-specific RNA hybridizing to radioactive cloned DNA probe was estimated by first comparing the intensity of the autoradiographic signal from the RNA dots to the DNA dots in the standard curve. Then, the nitrocellulose filter dots containing RNA:DNA or DNA:DNA hybrids were excised and counted on a scintillation counter. The counts obtained from the RNA dots were compared to the counts obtained for the known concentration of DNA in the standard curve. If several concentrations of RNA were spotted onto the filters, the count from each concentration was normalized to the amount of specific RNA/µg of total RNA and the different values were averaged to determine the amount of specific RNA/µg of total RNA.

**Methylation**—Aliquots of DNA (20 µg) prepared from pituitary tumors, nonestrogen-treated pituitaries, or DES-treated liver were digested to completion with MspI, HpaII, or HhaI. MspI and HpaII are isoschizomers that recognize the nucleotide sequence CCGG. HpaII cuts at this sequence only if the internal cytosine is unmethylated. MspI cuts at this sequence regardless of the methylation status of the internal cytosine. HhaI cuts at the sequence GCCG only if the internal cytosine is unmethylated. Restriction endonuclease-cleaved DNA was fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to specific fragments of DNA from the prolactin gene domain as described above.

**RESULTS**

**Tissue-specific DNAase I Sensitivity of the Prolactin Gene**—The prolactin gene is transcribed only in lactotrophs, a subpopulation of the five major cell types that comprise the anterior pituitary gland. The other major cell types (somatotrophs, corticotrophs, thyrotrophs, and gonadotrophs) normally represent 90–95% of the cells in intact anterior pituitaries of adult male rats (32). F344 rats, when chronically treated with the synthetic estrogen DES, develop pituitary tumors (18, 19) due to the predominant proliferation of lactotrophs (10). After 3 months of DES treatment, we observed tumors approximately 30 times the size of normal pituitaries (data not shown), confirming previous publications (18, 33). In Fig. 1a, a partial restriction map is shown of the recombinant clones containing the prolactin gene (34). Two exons and four introns, extending over approximately 10 kb of DNA, comprise the prolactin gene. Twelve kb of DNA 5‘ and 15 kb of DNA 3‘ to the prolactin gene also are included in these recombinant clones. Fig. 1b shows the location and size of specific restriction fragments referred to in the text and figures. Fig. 1c shows restriction fragments of prolactin DNA that were excised from the plasmid pBR322 by restriction enzyme cleavage and isoedicated by preparative agarose gel electrophoresis. These fragments were labeled to high specific activity by nick translation and used as probes in hybridization assays. The probes are identified in the text and figures as probe A, probe B, etc. according to the letter designation given in Fig. 1c.

Nuclei from pituitary tumors of liver of DES-treated animals were digested with various concentrations of DNase I at


**Fig. 1. Restriction map of the prolactin gene.** a, partial map of restriction endonuclease cleavage sites on 37 kb of cloned prolactin domain DNA (symbols are defined at the bottom). Filled boxes represent exons. b, location and size in kb of specific DNA fragments referred to in the text and figures. c, DNA fragments labeled by nick translation in the presence of [α-32P]dNTPs and used as probes in hybridization assays.

37°C for 10 min. DNA was purified, cut with restriction endonucleases, separated on 1% agarose gels, transferred to nitrocellulose (Southern), and hybridized to labeled prolactin restriction fragments or serum albumin cDNA.

Fig. 2, a and b, shows MspI- and HindIII-cutt DNA, respectively, from DNase I-digested pituitary tumor nuclei digestion. The DNA in Fig. 2a was hybridized to probe E1 (see Fig. 1c), and the DNA in Fig. 2b to the serum albumin cDNA. As expected from the restriction map, probe E1 hybridizes to two DNA fragments in the control lane. With increasing concentrations of DNase I, both prolactin bands are digested at approximately the same rate. As a control, the serum albumin gene in the same DNA preparation was digested only slightly, even at the highest concentration of DNase I (Fig. 2b).

Alternatively, Fig. 2, c and d, shows EcoRI-cut DNA from DNase I-digested liver nuclei. The DNA in Fig. 2c was hybridized to probe E3, and the DNA in Fig. 2d was hybridized to the serum albumin cDNA. In the liver, the prolactin gene is not highly sensitive to DNase I, but the serum albumin gene, in the same DNA preparation, is highly sensitive. Identical results as those shown in Fig. 2, a-d, were obtained when a nitrocellulose filter blot that had first been hybridized to either the serum albumin cDNA or probe E1, was stripped of the first probe by boiling 2 min in water, and rehybridized to the other hybridization probe (data not shown). The persistence of faint bands in the DNase I-sensitive genes, at all concentrations of DNase I used to digest chromatin, is probably due to the heterogeneous nature of the pituitary tumor or liver cell populations with respect to prolactin or serum albumin production, respectively.

Relative DNase I Sensitivity of Chromatin Throughout 35 kb of the Prolactin Gene Domain—Repetitive and unique sequences of the prolactin gene domain have previously been identified (see Fig. 7 for summary; Ref. 34). We used cloned 32P-labeled unique sequence DNA fragments from the 5′-flanking, coding, or 3′-flanking regions of the prolactin gene as hybridization probes. Aliquots of purified DNA from DNase I-treated pituitary tumor nuclei were cut to completion with a restriction endonuclease, separated by agarose gel electrophoresis, and transferred to nitrocellulose. A series of such filter blots was prepared and hybridized to different labeled DNA fragments. The DNA in Fig. 3, a and b, was cut with BamHI and HindIII, respectively. DNA in Fig. 3a was

**Fig. 3. Comparative DNase I sensitivity of 35 kb of prolactin domain chromatin in pituitary tumors.** Nuclei were digested 10 min with various concentrations of DNase I as shown at the top of the figure (micrograms/ml). Aliquots of purified DNA were restriction endonuclease-cleaved with BamHI (a), HindIII (b and d), MspI (c), or EcoRI (e and f). DNA fragments were separated by electrophoresis on 1% agarose gels and transferred to nitrocellulose. DNA on each filter was hybridized as follows: probe A (a), probe C (b), probe E1 (c), probe G (d and e), and probe N (f). The sizes of restriction fragments are given in kb and were determined as described in the legend to Fig. 2. DNA in the lane marked 10 μg/ml was digested by DNase I only to an extent equivalent to 3-5 μg/ml. The arrow in b indicates a sub-band that results from double-stranded cleavage of DNA by DNase I at a specific region in prolactin chromatin.

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hybridized to probe A$_2$ and DNA in Fig. 3b to probe C. Under these conditions, DNA regions 5' to the prolactin gene are detected. The 4.5-kb restriction fragment in Fig. 3c is situated greater than 7 kb upstream from the first exon of the prolactin gene (see Fig. 1b for location of fragments). The 6.9-kb restriction fragment in Fig. 3b extends immediately upstream from the first exon. The chromatin associated with these DNA fragments exhibits the same relative sensitivity to DNase I digestion; DNA is digested extensively at a DNase I concentration greater than 3.0 $\mu$g/ml. This is similar to the DNase I sensitivity of the chromatin associated with the coding region of the prolactin gene, shown in Fig. 3, c and d. DNA in Fig. 3c was cut with MspI and hybridized to probe E$_2$; DNA in Fig. 3d was cut with HindIII and hybridized to probe G. In both cases, the chromatin was digested at a DNase I concentration greater than 3.0 $\mu$g/ml. The restriction fragments in Fig. 3, e and f, are situated predominantly 3' to the prolactin gene. These DNA fragments exhibit the same relative sensitivity to DNase I digestion as the 5' and coding regions of the prolactin gene. The 7.3-kb EcoRI fragment in Fig. 3e, detected by hybridization to probe G, contains only the last exon of the prolactin gene. The remainder of this fragment is 3' sequence. The EcoRI fragment in Fig. 3f, detected by hybridization to probe N, is greater than 7 kb 3' to the prolactin gene. Genomic DNA homologous to probe N is present in several copies in the genome, hence the presence of several bands in this autoradiogram. The smallest band corresponds to the fragment size expected for prolactin DNA based on the restriction map of cloned DNA. Interestingly, all three restriction fragments observed exhibit the same sensitivity to DNase I, suggesting a similar chromatin structure of these fragments in the genome. When chromatin was digested with a narrower concentration range of DNase I than shown here, the same results were observed; the prolactin gene is situated in at least 35 kb of DNA chromatin that exhibits a uniform sensitivity to DNase I digestion.

As a control for these experiments, DNA from these same DNase I digests was cleaved with EcoRI and hybridized to the serum albumin cDNA. The serum albumin gene was insensitive to DNase I digestion even at a nucleosome concentration as high as 20 $\mu$g/ml (data not shown).

Localization of Hypersensitive Sites near the Prolactin Gene—The sub-band observed in Fig. 3b (arrow) is a DNase I-hypersensitive site that results when DNase I cuts both strands of DNA at a specific site in chromatin. This hypersensitive site is situated approximately 1.8 kb upstream from the transcription start site in a region that contains repetitive sequences (see Fig. 7). We examined this site further by cleaving genomic DNA from DNase I-digested chromatin to completion with XhoI, and then hybridizing to probe E$_2$, which represents DNA situated 3' to the hypersensitive site. Under these conditions, we observed the 3.8-kb XhoI restriction fragment and, in addition, two sub-bands (Fig. 4, third lane) which are observed only in samples treated with DNase I (Fig. 4, compare second and third lanes). The presence of two sub-bands indicates that two hypersensitive sites are located near the prolactin gene. The second hypersensitive site maps about 150 base pairs upstream from the transcription start site. These DNase I-hypersensitive sites are not observed in liver chromatin (Fig. 4, first lane). We are currently characterizing these sites more thoroughly. No other hypersensitive sites were detected in the 35 kb of prolactin chromatin examined (Fig. 3, a-f, and results confirmed by mapping experiments using different restriction endonucleases and the hybridization probes used here; data not shown).

Correlation of DNase I Sensitivity with Transcriptional Ac-
FIG. 5. RNA transcripts homologous to specific fragments of cloned prolactin DNA. Nuclear or cytoplasmic RNA from pituitary tumors or liver was spotted onto nitrocellulose filters in the amounts shown in the key. Known amounts of prolactin-specific DNA were spotted onto the same filters, as were 5 μg of λDNA and 5 μg of E. coli tRNA, for controls. The letters in the right lower corner of each panel refer to the prolactin DNA fragment from Fig. 1c to which the blots are hybridized. RNA in the panel marked SA was hybridized to the serum albumin cDNA.

regions 5' or 3' to the prolactin gene were detected (A₂, B, C, or N) in either pituitary tumors or liver (faint dots are seen for 5 μg of liver nuclear RNA, but the radioactivity in these dots, determined by scintillation counting, was no higher than counts in the control dots). The dot blot hybridization assay used for this work can detect less than 1 pg of homologous RNA in 5 μg of total RNA (31). However, we cannot exclude the possibility that these DNA sequences are transcribed but the transcripts are rapidly degraded. We examined this by fractionating cytoplasmic and nuclear RNAs on formaldehyde gels. After the RNA was transferred from the gel to a nitrocellulose filter and hybridized to probe E₁, we were able to detect nuclear precursors of the prolactin mRNA (which are rapidly processed). Alternatively, if the blot was hybridized instead to probe C, we could not detect transcripts homologous to unique DNA sequences outside the prolactin gene (data not shown). We conclude that transcripts homologous to unique DNA sequences 5' or 3' to the prolactin gene must account for less than 10⁻⁶ of the total RNA.

Table I shows an estimate of the picograms of RNA/μg of total RNA, homologous to specific prolactin restriction fragments. The data in this table were obtained by excising the spots from the nitrocellulose filters, whose autoradiograms are shown in Fig. 5, and quantitating the radioactivity as described under "Materials and Methods." These estimates of the number of homologous RNA transcripts are only semi-quantitative due to the limited accuracy of the dot blot hybridization assay. However, this dot blot hybridization assay is valuable for estimating the relative abundance of one transcript as compared to another. As expected, a high concentration of prolactin transcripts is present in both the cytoplasm and nucleus of pituitary tumors but not in liver (E₁ and G). Transcripts homologous to the fourth intron of the prolactin gene (F), which contains Alu-like repeats (34), are not detected in the cytoplasm and are present in low concentration in the nucleus. This is expected since introns are usually processed and degraded rapidly. This repetitive sequence does not appear to be transcribed abundantly elsewhere in the genome. The repetitive sequences A₁, I, J, and K hybridize to homologous nuclear RNA transcripts. Unexpectedly, probe A₁ also hybridizes to transcripts present in the cytoplasm. A similar result was obtained for liver RNA. Transcripts ho-
mologous to repetitive probes A₁, F, H, I, J, K, and L were present in high concentration in liver nuclear RNA. But only probe A₁ hybridized to cytoplasmic RNA. Serum albumin transcripts are present in high levels only in the cytoplasm and nucleus of liver.

**Tissue-specific Hypomethylation of the Prolactin Gene**—Transcribed DNA sequences are generally unmethylated, while adjacent nontranscribed DNA sequences are methylated. Hypomethylation of a gene is tissue-specific. We used the restriction endonucleases MspI, HpaII, and HhaI to define the methylation pattern of the prolactin gene in DES-induced pituitary tumors, control pituitaries, and liver. Fig. 1a and 6e show the MspI/HpaII and HhaI restriction sites in the prolactin gene domain.

Genomic DNA was digested with MspI, HpaII, or HhaI, fractionated in adjacent lanes by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to one of the labeled unique sequence restriction fragments shown in Fig. 1c. When MspI-cleaved DNA is hybridized to probe E₁, 4.6- and 1.8-kb bands are observed (Fig. 6b, first, fourth, and seventh lanes). A 6.4-kb band is never seen, indicating that whenever the two outer sites are unmethylated, the inner site is also unmethylated. In liver, no discrete bands are observed for HpaII-cleaved DNA (Fig. 6b, eighth lane), suggesting the three MspI/HpaII sites within the coding region of the prolactin gene are methylated (this experiment does not prove conclusively, though, that all three sites are methylated). In control pituitaries and pituitary tumors, however, both bands are observed (Fig. 6b, second and fifth lanes). A 10.8-kb band is observed when pituitary tumor, but not control pituitary or liver DNA, is digested with HhaI and hybridized to probe E₁ (Fig. 6b, sixth, third, and ninth lanes, respectively). The autoradiographic intensity of the HhaI band is less than for the HpaII band. These results indicate that the HhaI sites located immediately 5' and in the last exon of the prolactin gene are at least partially unmethylated in lactotrophs; the lesser intensity of the HhaI band as compared to the HpaII band could result if one or both of these HhaI sites are methylated in a subset of the DNA molecules examined but the HpaII sites in the same DNA molecules are unmethylated. The 10.8-kb HhaI restriction fragment in control pituitary would not have been observed if only a few per cent of the DNA molecules were unmethylated at this site (see below). Alternatively, this site may be methylated in control pituitaries, and estrogen induces demethylation of this site.

When MspI-cleaved DNA is hybridized to probe C, a 10.9-kb band is observed (Fig. 6a, first, fourth, and seventh lanes). When pituitary tumor, control pituitary, or liver DNA is digested with HpaII, no discrete bands are found (Fig. 6a, second, fifth, or eighth lane). Thus, both MspI/HpaII sites 5' to the prolactin gene are methylated in all tissues studied. When HhaI-cleaved DNA from either of the three tissues is hybridized to probe C, no discrete bands are observed (Fig. 6a, third, sixth, or ninth lane). HhaI sites further 5' than the one nearest the first exon must be methylated.

Hybridization of MspI-cleaved DNA to probe N results in a complex pattern of bands (Fig. 6c, first, fourth, and seventh lanes) due to the repetitive nature of this sequence in the genome. The restriction map of cloned prolactin DNA indicates that the prolactin bands are 12.5, 3.5, and 0.6 kb in length. Only the larger two bands are actually observed, however, possibly due to the poor binding of smaller DNA fragments to nitrocellulose filters after transfer from agarose gels. When probe N is hybridized to HpaII- or HhaI-cleaved DNA from pituitary or liver, no discrete bands are seen (Fig. 6c, second, third, fifth, sixth, eighth, and ninth lanes). The 3' region of the prolactin gene domain is, like the 5' region, methylated.

The method we employed above can detect the presence of methylation when only 10–20% of the DNA sequences in a DNA sample are methylated (14). To determine the extent of methylation of the transcribed region of the prolactin gene in DES-induced pituitary tumors, DNA was prepared from rats treated with DES for various times from 0 to 15 weeks. Aliquots of these purified DNAs were digested with HpaII and SstI. SstI cleaves DNA regardless of the methylation pattern. The DNA was then prepared for hybridization as described under "Materials and Methods." When DNA is digested with SstI only and hybridized to probe E₁, a 7.8-kb band is detected (see Fig. 6e for restriction map). HpaII cleaves prolactin DNA three times within the SstI restriction fragment, resulting in the appearance of three bands (Fig. 6d, second to fifth lanes). The 7.8-kb band would be observed after HpaII/SstI digestion only if some of the prolactin genes
in a population of DNA remain methylated. The autoradiographic intensity of the three HpaII bands, as compared to the SstI band, indicates the relative extent of methylation of the prolactin gene. In Fig. 6d, the DNA in the first lane was digested with MspI. Two major bands are observed at 4.6 and 1.8 kb (as well as fainter bands resulting from partial digestion). The other lanes contain HpaII/SstI-cleaved prolactin DNA from F344 rats treated with DES for the number of weeks shown. With increasing time of DES treatment, there is an increase in the proportion of prolactin DNA that is unmethylated.

**DISCUSSION**

We have summarized in Fig. 7 the methylation pattern, location of repetitive DNA sequences, and DNase I-hypersensitive sites associated with the prolactin gene domain in anterior pituitary tumors of F344 rats. In addition, this entire chromatin domain (35 kb) is sensitive (as compared to liver chromatin) to DNase I digestion.

In several systems, acquisition of a DNase I-sensitive chromatin conformation has been demonstrated to occur during differentiation (12, 35, 36). Only in the *Xenopus* vitellogenin gene chromatin system has a steroid (estrogen) been shown to directly induce a more DNase I-sensitive conformation (37). Demethylation of transcribed DNA sequences likewise has been shown to occur during differentiation (12, 13); and, in a few cases, a specific MspI/HpaII site becomes hypomethylated upon administration of a steroid (38, 39).

Estrogen induces an increase in prolactin gene transcription (3, 4), and estrogen may also be involved in differentiation of lactotrophs (40). The F344 pituitary tumors we used to study the prolactin gene chromatin are induced by chronic estrogen treatment. Therefore, we were not able to determine whether differences we observed in chromatin conformation and DNA structure of the prolactin gene result from a direct effect of estrogen or whether they result from other processes occurring during differentiation. Very likely, in the F344 pituitary system we employed, estrogen simply causes proliferation of fully differentiated lactotrophs. The prolactin genes in the tumor cells then retain the chromatin and DNA structures associated with their progenitor cells. Some support of this idea is given by the methylation data we presented, especially Fig. 6d. In direct correlation with an increase in the proportion of lactotrophs (33), the percentage of hypomethylated prolactin genes increases.

DES obviously does not induce demethylation of MspI/HpaII sites near the prolactin gene in tissues not destined to produce prolactin. Even after 3 months of chronic estrogen treatment, a fraction of the prolactin genes in the anterior pituitary and all those in liver are methylated. Estrogen may induce demethylation of one or both of the HhaI sites within the first exon, or immediately 5' to the prolactin gene in lactotrophs, although we have not shown this conclusively.

The coding region of the prolactin gene exhibits the same degree of sensitivity to DNase I as nontranscribed regions up to 12 kb upstream or downstream. In contrast, early reports in the literature showed that the coding regions of the β-globin genes (13), α-globin genes (12), and ovalbumin gene (14) of the chicken, as well as integrated adenovirus genes in hamster cells (41), are highly sensitive to digestion by DNase I, whereas noncoding regions several kb upstream or downstream of these genes display an intermediate sensitivity. Subsequently, Wood and Felsenfeld (15) and, more recently, Nicolas et al. (16) found no differential DNase I sensitivity between coding and noncoding regions of the β-globin gene. Lawton et al. (17) studied the chromatin structure of the ovalbumin and X and Y genes of the chicken and found approximately 100 kb of DNA within and around these genes in a uniformly DNase I-sensitive conformation. The latter group suggested that the high level of DNase I digestion they employed might have obliterated more labile DNase I-sensitive structures detected previously by limited DNase I nicking and filter blot hybridization (12-14). Subsequently, Wood et al. (16) presented evidence that supports this claim. They detected a differential sensitivity of the β-globin gene and surrounding sequences, after limited DNase I nicking of chromatin and blot hybridization, but not by solution hybridization after extensive DNase I digestion. Flint and Weirntraub (41), however, extensively digested chromatin with DNase I and showed by solution hybridization that the highly DNase I-sensitive adenovirus genes, integrated into hamster cell DNA, are bordered by relatively DNase I-insensitive regions a few nucleosomes upstream or downstream.

Differential sensitivity to DNase I digestion between the coding and noncoding regions of a gene does not appear to be a universal phenomenon and is not consistently observed in chromatin of a single gene (i.e. ovalbumin or β-globin genes of the chicken). If such differences in DNase I sensitivity of coding and noncoding regions of a gene domain do exist, our inability to detect this difference in the prolactin gene domain may be due to the presence of other transcription units around this gene. However, we were not able to detect transcripts homologous to any of the unique sequence regions.

We did not detect 5' or 3' boundaries to the DNase I-sensitive chromatin region, as have been previously reported for the ovalbumin gene family (17) and for the α- and β-globin genes (12, 13). Possibly such DNase I-insensitive regions do exist farther 5' or 3' from the prolactin gene than examined here. It has been suggested that large DNase I-sensitive regions found in chromatin may be analogous to the 30-90-kb DNA loops observed after deproteinization of metaphase chromosomes (16) or of lambrush chromosomes (13).

Chromatin hypersensitive sites have been localized to regions 5' to the α- and β-globin genes (12, 13), *Drosophila* heat shock genes (8), vitellogenin gene (38), as well as many other genes (see Ref. 42 for review). These sites have often been localized to specific DNA sequences near the transcription start site of genes; additional hypersensitive sites have been observed further upstream from the vitellogenin gene of the chicken (38) and the glue protein genes of *Drosophila* (43). Hypersensitive sites have occasionally been observed 3' to genes or in regions not known to be regulatory regions (42). The precise role of hypersensitive sites is not known, but they
are closely correlated with transcriptional activity (8, 44). In addition, three hypersensitive sites 5' to the chicken vitellogenin gene are induced by estrogen (38). Two of these sites persist after estrogen removal, but the other site is present only during estrogen treatment.

The two hypersensitive sites 5' to the prolactin gene were identified in estrogen-treated pituitaries; therefore, we do not know if either of these hypersensitive sites might be induced in chromatin by estrogen. These sites warrant further study as possible transcription regulatory sites due to their location 5' to the prolactin gene.

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