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# Significance of the Chromatin Structure in Expression of the Rat Prolactin Gene[1]

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**To elucidate the mechanisms underlying cell type-specific expression of the growth hormone (GH) and prolactin (PRL) genes, we used rat pituitary-derived cell lines producing exclusively GH (GC cells) or PRL (235 cells), and examined the following: expression of transcription factors essential for GH and/or PRL gene expression; promoter/enhancer activity of the GH and PRL genes transiently introduced by transfection; and chromatin structures of the GH and PRL genes. Even in PRL-nonproducing GC cells, the PRL promoter/enhancer was more active than the GH promoter, and the transcription factors, Pit-1 and estrogen receptor (ER), essential for PRL gene expression were functional. The PRL promoter/enhancer of GC cells was normal. On DNase I sensitivity analysis of the chromatin structure, two hypersensitive sites were detected in PRL gene chromatin of PRL-producing 235 cells but none in that of GC cells. It thus follows that the major reason for absence of the expression of the endogenous PRL gene in GC cells is neither the lack of transcription factors necessary for PRL gene expression nor an anomaly of the PRL gene itself, but that the chromatin structure of the PRL gene differs in PRL-nonproducing and -producing cells. It was shown in this study that neither Pit-1 nor ER is required for conversion of the structure of PRL gene chromatin to a DNase I-hypersensitive state.**

**Key words: chromatin structure, DNase I-hypersensitivity, gene expression, prolactin, transcription factor.**

Growth hormone (GH) and prolactin (PRL) are vertebrate pituitary hormones each consisting of 180 to 200 amino acid residues. The similarities in amino acid sequence and gene organization suggest the genes for these hormones are possibly derived from a common ancestor (*1*). GH and PRL are produced in somatotrophs and lactotrophs, respectively. The expression of GH and PRL in these tissues has been studied as a model system for elucidating the mechanism underlying cell type-specific gene expression in vertebrates. Studies on transcription factors and *cis*-elements led to the discovery of a pituitary-specific transcription factor, Pit-1/GHF-1 (referred to as Pit-1 in this article) (*2, 3*), which participates in the cell type-specific expression of the GH and PRL genes. Pit-1 stimulates transcription of the rat GH gene by binding to two specific sites situated within 200 bp upstream from the transcription start site. Several rat Pit-1 binding sites, four of which are located within 200 bp upstream from the transcription start site of the rat PRL gene and an additional four in the 1.5- to 1.8-kb upstream region, are involved in PRL gene expression (*4*).

After Pit-1, the T3 receptor (T3R) is the most well-studied transcription factor of the rat GH gene acting on the *cis*-element (thyroid hormone responsive element, TRE) located about 0.2 kb upstream from the transcription start site (*5*). The action of the estrogen/estrogen receptor (ER) toward the binding element (estrogen responsive element, ERE) located just downstream from the four Pit-1 binding sites in the PRL distal enhancer is another case (*6*).

To understand the mechanism underlying cell type-specific expression of the GH and PRL genes, studies on transcription factors and their interacting *cis*-elements are surely important. However, it would be particularly significant to clarify the structural features of chromatin, that serves as the template for transcription (*7, 8*). For expression of the GH or PRL gene in pituitary organogenesis, it would appear necessary for the chromatin structure of the GH or PRL gene to change from a condensed to a relaxed state, where the transcription factor interacts with cognate *cis*-elements. But nothing is known about the mechanism by which the chromatin structure of a particular gene and its flanking regions is loosened in the course of development and differentiation. The chromatin of an actively transcribed gene is sensitive to DNase I digestion, and cell type-specific DNase I-hypersensitive site(s) (DHS) are often found in and around the gene (*9, 10*). As judged mostly from studies of transgenic mice, the region corresponding to cell type-specific DHS found 10 to 20 kb upstream from the human β-globin gene cluster serves to loosen the chromatin structures of gene clusters in the expression of

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Abbreviations: DHS, DNase I-hypersensitive site(s); ER, estrogen receptor; ERE, estrogen responsive element; GH, growth hormone; LCR, locus control region; PRL, prolactin; T3R, T3 receptor; TRE, thyroid hormone responsive element.



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this gene family (*11, 12*).

In PRL-producing cells, at least a 35 kb region including about 12 kb upstream and downstream regions of the rat PRL gene was found to be totally DNase I-sensitive, and two cell type-specific DHS were noted to be located at 0.1 and 1.65 kb upstream from the transcription start site (*13*). These DHS corresponded well with the PRL promoter and distal enhancer, each having four Pit-1 binding sites (*4*), and thus, Pit-1 and/or ER may function to loosen PRL gene chromatin and generate DHS.

GC, 235, and GH3 are cell lines derived from rat pituitaries (*14*). GC and 235 cells exclusively produce GH and PRL, respectively, whereas GH3 produces both GH and PRL. To elucidate the mechanism underlying the cell type-specific expression of the GH and PRL genes in these cells, the following were examined: expression of transcription factors essential for GH and/or PRL gene expression, by Northern hybridization; promoter/enhancer activity of the GH and PRL genes, by transient transfection; and the chromatin structures of GH and PRL gene loci, by DNase I-sensitivity analysis.

## MATERIALS AND METHODS

*Cells, RNA, and Northern Hybridization*—GC, GH3, and NRK (rat kidney fibroblast) cells were grown as described (*14*). 235 cells were grown in Dulbecco's modified minimal essential medium/F12 containing 5% fetal bovine serum (JRH Biosciences, USA) and 5% horse serum (JRH Biosciences) plus 10 nM $\beta$-estradiol due to their slower growth rate without estrogen. The concentrations of 3,5,3'-triiodothyronine (T3), thyroxine (T4), $\beta$-estradiol (E2), and hydrocortisone in the medium for pituitary cells were 0.2 nM, 9 nM, <4 pM, and 16 nM, respectively, as determined by RIA (Sumitomo Bioscience, Sagamihara). To examine the effects of exogenously added thyroid or steroid hormones on GH, PRL, T3R, and ER gene expression in pituitary cell lines, the cells were grown in the presence of 10 nM T3, 10 nM E2, or 1 $\mu$M dexamethasone (Dex) for at least 1 week. Total RNA and poly(A)$^+$ RNA were prepared as described previously (*14*). RNA was denatured, fractionated in a 2.2 M HCOH/1% agarose gel, transferred to nitrocellulose filter, and then hybridized with probes as previously described (*14*). Following hybridization, the filter was washed in 0.1×SSC (1×SSC: 150 mM NaCl/15 mM $Na_3$·citrate)/0.1% SDS at 65°C. Filter reprobing with a human glyceraldehyde 3-phosphate dehydrogenase cDNA (G3PDH) probe (Clontech) was conducted according to the instructions provided. Using rat pituitary cDNA as the template (*15*), rat GH (nt 18-698) (*16*), and PRL (nt 32-763) (*17*) cDNAs were amplified by PCR and then subcloned into Bluescript. Using *Pfu* polymerase (New England Biolab., Beverly, USA), rat pituitary T3R$\beta$-1 (nt 256-1646) (*18*), T3R$\beta$-2 [nt 47-494 (*19*), nt 530-1646 (*18*)], and ER (nt 200-2022) (*20*) cDNAs were amplified by PCR and then subcloned into pRc/RSV (5,133 bp, Invitrogen, San Diego, USA). As hybridization probes, T3R$\beta$-1 (nt 256-463), T3R$\beta$-2 (nt 47-384), and ER (nt 1226-2022) were used. Cloned DNA was labeled by the random primed method using [$\alpha$-$^{32}$P]dCTP.

*Transfection*—The Pica Gene basic vector, PGV-B (5,597 bp; Toyo Ink, Tokyo), possessing the firefly luciferase (Luc) gene, served as the reporter gene. Luc activity was assayed using a Pica Gene Luminescence kit, PKG-100 (Toyo Ink). The reporter genes having rat GH (−318 to +12, GH-P) and PRL (−450 to +36, PRL-P) promoter regions were as described previously (*14*). The rat PRL distal enhancer containing the −1800 to −1447 region (PRL-E) (*21*) was constructed by the PCR method and cloned into the *Kpn*I site of the PRL-P reporter (*22*). Reporter plasmids containing GH-P or PRL(P+E) of GC or 235 origin were prepared by the above method using GC or 235 DNA. Transfection was conducted by the lipofectin method (GIBCO-BRL) for 16 h as described previously (*14*). One day following medium renewal, luciferase activity was measured as previously described (*15*). A BCA protein assay kit (Pierce) was used for protein determination.

*Nuclease Digestion and Southern Hybridization*—Isolation of GC and 235 nuclei, DNase I digestion and Southern hybridization were carried out as previously described (*22*). For restriction enzyme digestion, a 270 $\mu$l reaction mixture containing $1\times10^7$ nuclei was incubated for 60 min at 37°C and the reaction was terminated with 25 $\mu$l of a stop solution. The rat PRL gene containing the −450 to +608 region (*17*) was prepared by PCR using male Wistar rat liver DNA and then cloned into Bluescript. A rat GH cDNA probe, nt 18-424, was prepared as described previously (*22*).

## RESULTS AND DISCUSSION

*Expression of T3R and ER mRNA in GC, 235, and GH3 Cells*—As reported previously (*14*), Northern hybridization revealed the exclusive production of GH and PRL mRNAs in GC and 235 cells, respectively, while GH3 cells produced both mRNAs under the cell culture conditions used in this study. The Pit-1 mRNA content was shown to be essentially the same in GC, 235, and GH3 cells (*14*), and thus factor(s) other than Pit-1 and/or differences in the chromatin structures of GH and PRL gene loci should be necessary for GH gene expression in GC cells and PRL gene expression in 235 cells.

In addition to those of Pit-1, the roles of T3R and ER in GH and PRL gene expression, respectively, have been studied extensively (*5, 6*). In this study, T3R and ER gene expression in these cells was also examined (Fig. 1). T3R$\alpha$-1 and T3R$\beta$-1 are present in most tissues (*23*), while T3R$\beta$-2 is characteristic of pituitary tissue (*19*). $\beta$-1 and $\beta$-2 mRNAs are isoforms generated from a single gene through alternative splicing. These receptors have the same DNA binding and T3 binding domains but different N-terminal regions (*19*). As shown in Fig. 1A, $\beta$-1 mRNA was expressed in three pituitary-derived cells, but $\beta$-2 mRNA only in GH-producing GC and GH3 cells (Fig. 1B). Forman *et al.* reported the absence of T3R in 235-1 cells and thus the discrepancy remains to be explained (*24*). All three pituitary-originating cells contained basically the same amount of ER mRNA (Fig. 1C). Neither T3R$\beta$-1, T3R$\beta$-2, nor ER mRNA was found in rat kidney-derived NRK cells.

*Effects of Thyroid Hormone and Estrogen on GH, PRL, T3R, and ER Gene Expression in GC, 235, and GH3 Cells*—In hormone-producing cells in the pituitary gland, thyroid hormone and glucocorticoid stimulate transcription of the GH gene, while E2 stimulates PRL gene transcription. Although certain amounts of thyroid hormone, cor-



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tisol, and E2 are present in sera used in cell culture media for pituitary cell lines, they may not be adequate for full production of GH and PRL in these cells. Thus, the effects of T3, dexamethasone (Dex), and E2 on GH and PRL production were examined by adding them at optimal concentrations. The expression of T3R and ER mRNAs under the same conditions was examined (Fig. 2). T3 and Dex stimulated the expression of GH mRNA in GH-producing cells, as reported previously. E2 increased the PRL mRNA content of GH3 cells but partially decreased that of 235 cells. The expression of T3Rβ-1 and T3Rβ-2 mRNAs was relatively constant in GC, 235, and GH3 cells under the present conditions. A slight decrease in the ER mRNA content was observed in 235 cells treated with T3+Dex+E2 or T3+Dex, whereas an increase in the content of smaller mRNA was found in GH3 cells with this treatment. The contents of GH and PRL mRNAs in GC, 235, and GH3 cells under the conditions used showed no great changes.

*Activity of Transiently Introduced GH and PRL Promoter/Enhancer in GC and 235 Cells*—Since the contents of Pit-1 and ER mRNAs, both being required for PRL gene expression, in PRL-nonproducing GC cells were similar to those in PRL-producing cells, assessment was made of the activity of PRL promoter/enhancer, and Pit-1 and ER activity in GC cells was confirmed by the transient transfection method using PRL promoter/enhancer (Fig. 3). Transiently introduced GH promoter (GH-P) was active in GC cells, thus suggesting Pit-1 mRNA was translated into the functional Pit-1 protein in these cells. Although PRL promoter (PRL-P) was less active than GH-P, possibly due to the cell type-specificities of the promoters (*14, 25*), PRL-P in combination with the distal enhancer, PRL(P+E), showed higher activity than GH-P. In 235 cells, the activity of PRL-P was higher than that of GH-P, as reported previously (*14, 25*), and was again found to be maximum in combination with the distal enhancer. Not only Pit-1 but also ER have been shown to be involved in this 18-fold stimulation. The stimulation ratio of PRL(P+E)/PRL-P (13-fold) in GC cells was similar to that in 235 cells, thus suggesting ER in GC cells is as active as that in 235 cells.

As judged from this and other studies, GH-P shows much less activity than PRL(P+E) in PRL-producing cells (Fig. 3B) (*14, 25*), possibly due in part to the absence of T3Rβ-2 in 235 cells (Fig. 1B). Since no significant change in GH-P activity was observed on co-transfection of the T3Rβ-2 effector plasmid having the RSV-LTR promoter with the GH-P reporter in 235 cells (M. Ono, unpublished data), the reason for the poor GH-P activity in 235 cells would not be the absence of T3Rβ-2.

The reason for the absence of expression of the PRL gene in GC cells may not be an anomaly of the PRL gene itself. For confirmation of this, reporter plasmids containing the GC-derived PRL(P+E) region amplified by PCR were introduced into GC cells (Fig. 3C). The activity of the reporters was basically the same as that of 235- and normal rat-derived reporters, thus showing the PRL(P+E) region in GC cells to be normal. The 235-derived GH promoter region was again shown to be as active as the GC-derived region, thus indicating it to be normal in 235 cells.

*Nuclease Sensitivity of PRL and GH Gene Chromatin in 235 and GC Cells*—To determine the structures of PRL and GH gene chromatin, which functions as templates in transcription, DNase I-hypersensitive site(s) (DHS) mapping was carried out (Figs. 4 and 5). Within an interval comprising a 12 kb upstream region, 10 kb gene region and 12 kb downstream region of the rat PRL gene, only two DHS, at 0.1 and 1.65 kb upstream from the transcription start site, have been reported in rat PRL gene chromatin of

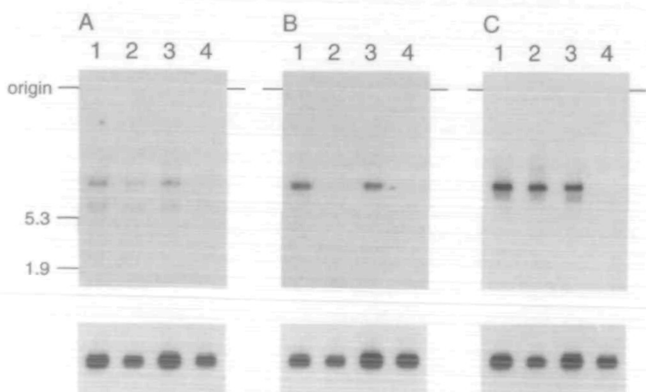


Fig. 1. **Expression of T3R and ER mRNA in GC, 235, and GH3 cells.** Poly(A)+ RNA (0.8 μg) was denatured and electrophoresed on a 2.2 M HCOH/1% agarose gel, and then hybridized with ³²P-labeled rat T3Rβ-1 (A), T3Rβ-2 (B), or ER (C) cDNA. The numbers in the left margin are in kb. At the bottom, reprobing of A-C with G3PDH cDNA is shown Lanes: 1, GC; 2, 235; 3, GH3, 4, NRK

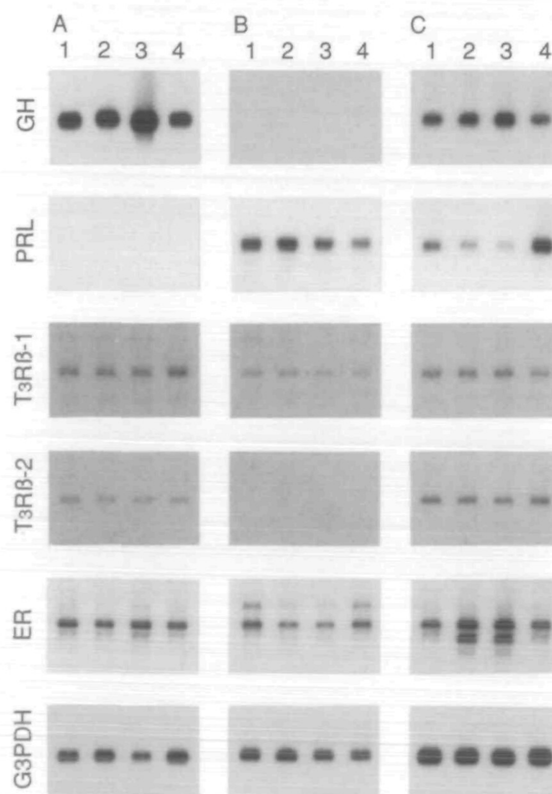


Fig 2 **Effects of T3, Dex, and E2 on GH, PRL, T3R, and ER gene expression in GC, 235, and GH3 cells.** Cells were grown in the presence of exogenously added 10 nM T3, 10 nM E2, or 1 μM Dex for at least one week Poly(A)+ RNA (0.8 μg) was analyzed as in Fig 1 with the ³²P-labeled probes shown on the left. At the bottom, reprobing of T3Rβ-2 hybridization with G3PDH cDNA is shown. A, GC, B, 235; C, GH3 1, none; 2, T3+Dex+E2; 3, T3+Dex; 4, E2.



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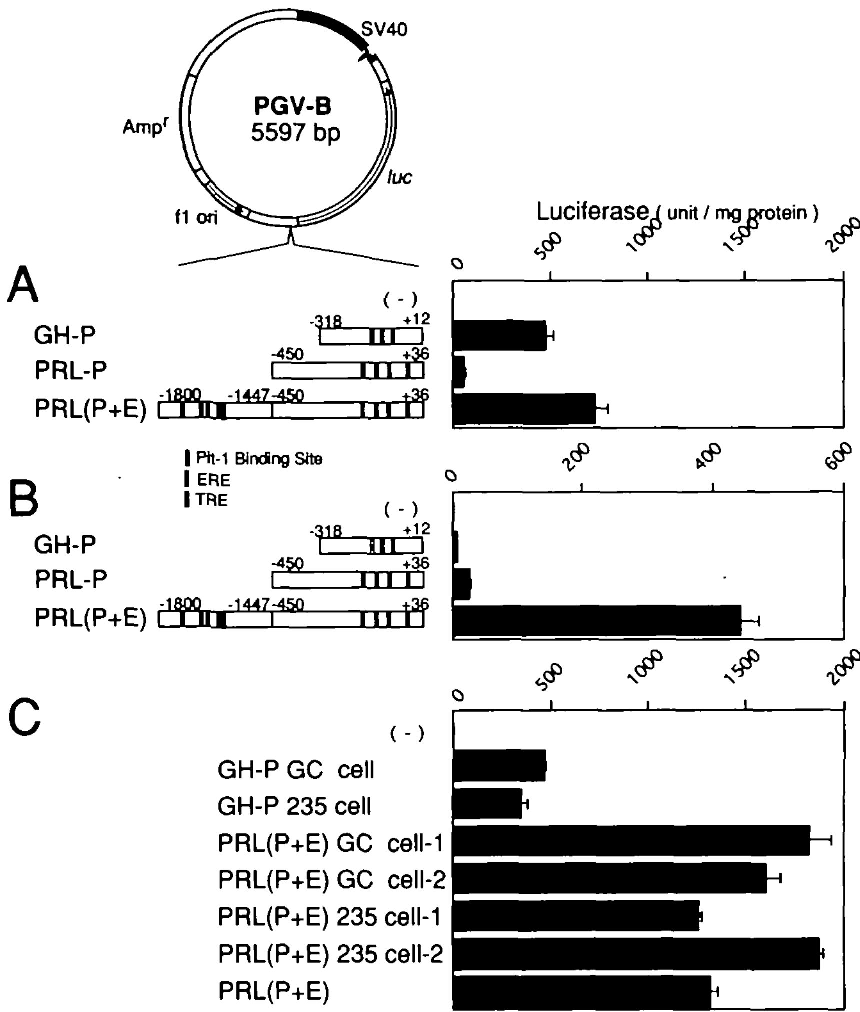


Fig. 3. **Activity of GH and PRL promoter/enhancer transiently introduced into GC and 235 cells.** The promoter and/or enhancer are shown on the left. Values are the means for triplicate samples (±SEM). A, GC; B, 235; C, GC. Cell-1 and cell-2 were independent constructs. (−), promoter minus (PGV-B); GH-P, GH promoter; PRL-P, PRL promoter; PRL(P+E), PRL-P plus distal enhancer. ERE, estrogen responsive element; TRE, thyroid hormone responsive element. One light unit of luciferase corresponds to $1.4\times10^{-17}$ mol Luc.

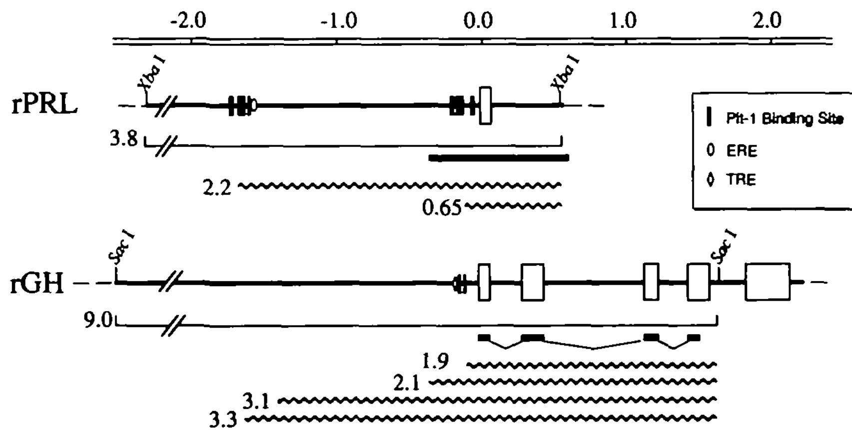


Fig. 4. **Structure near the transcriptional start sites of the rat PRL and GH genes.** The probes used for Southern hybridization are indicated. The fragments generated on DNase I digestion are shown by wavy lines. Sizes are indicated in kb. ERE, estrogen responsive element; TRE, thyroid hormone responsive element; open box, exon.

PRL-producing cells (*13*). In PRL-producing 235 cells, two DHS were found at basically the same sites as those reported by Durrin *et al.* (*13*) (Fig. 5A). We investigated the restriction enzyme sensitivities of PRL gene chromatin. These enzymes cut DNA at specific sites and thus all fragments will give distinct bands on autoradiography. Two bands (0.89 and 2.3 kb; Fig. 6A), corresponding to 0.65 and 2.2 kb, respectively, were detected on digestion of 235 nuclei with *Hin*fI, although limitation of the *Hin*fI cutting sites in the PRL gene caused the fragment sizes to differ slightly from those generated on DNase I cutting. No extra band was detected on digestion of PRL-nonproducing GC nuclei with DNase I (Fig. 5B) or *Hin*fI (Fig. 6B). The DNase I-sensitivity of PRL gene chromatin in GH3 cells was virtually the same as that in 235 cells (M. Ono, unpublished data).

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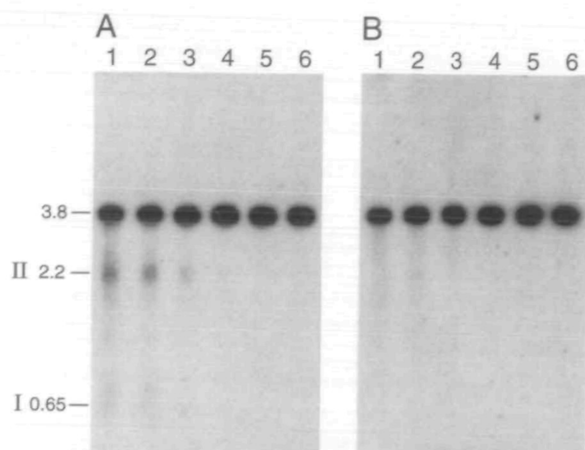


Fig. 5. **DNase I-sensitivity of PRL and GH gene chromatins.** Isolated nuclei were treated with the indicated amounts of DNase I for 3 min at 20°C. DNAs were purified from nuclei and digested with *Xba*I. The digests (3 $\mu$g) were separated by electrophoresis on 0.75% agarose gels and then blotted onto nitrocellulose filters. A, 235, B, GC. The blots were hybridized with rat PRL (−450 to +608). Restriction fragment sizes (given in kb) were determined with *Hin*dIII-cleaved $\lambda$ DNA. Concentrations of DNase I for treatment of nuclei (in $\mu$g/ml): lane 1, 2; lane 2, 1; lane 3, 0 5; lane 4, 0.25, lane 5, 0.13; lane 6, 0

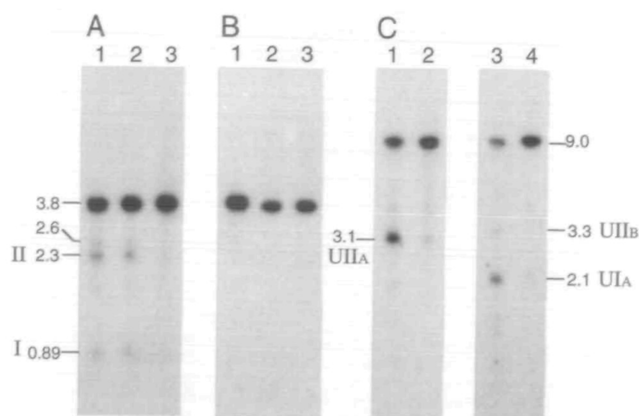


Fig 6. **Digestion of PRL and GH gene chromatins by restriction enzymes.** Nuclei were digested with the indicated amounts of *Hin*fI (A, B) or *Alu*I (C) for 60 min at 37°C DNAs were purified from nuclei and digested with *Xba*I (A, B) or *Sac*I (C) The digests (3 $\mu$g) were separated by electrophoresis on 0.75% agarose gels and then blotted onto nitrocellulose filters (A, C1, C2), 235; (B, C3, C4), GC. Blots A and B were hybridized with rat PRL (−450 to +608), and C with rat GH cDNA (+18 to +424) Restriction fragment sizes (given in kb) were determined with *Hin*dIII-cleaved $\lambda$ DNA Concentrations of restriction enzymes (u/ml): lanes A1, B1, 300; A2, B2, 150; A3, B3, 0, C1, C3, 150, C2, C4, 0.

Contrary to the DNase I-insensitivity of PRL gene chromatin in GC cells, GH gene chromatin was found to be sensitive (*22*). For example, in the upstream region of GH gene chromatin, a GH probe was found to be able to detect one major DHS (M-DHS) corresponding to a 2.1 kb fragment (UIA, at −0.35 kb from the transcription start site), and two minor DHS (m-DHS) corresponding to a 1.9 kb fragment (UIB, −0.15 kb) and a 3.3 kb fragment (UIIB, −1.6 kb). In 235 cells, M-DHS corresponding to a 3.1 kb fragment (UIIA, −1.4 kb) was generated on DNase I digestion of GH gene chromatin at high concentration (*22*). On *Alu*I digestion of GC and 235 nuclei, bands exhibiting the same mobilities as observed in the case of DNase I digestion were detected (Fig. 6C), though no 1.9 kb (IB) fragment was found since no *Alu*I site was present to generate this fragment in GC cells.

It follows from the above that the major reason for the lack of endogenous PRL gene expression in GC cells is not the absence of transcription factors necessary for PRL gene expression or an anomaly of the PRL gene itself, but differences in the chromatin structure of the PRL gene between in PRL-nonproducing and -producing cells. Thus, not only transcription factors but also factor-accessible chromatin should be prepared for expression of the PRL gene. Although some transcription factors derived from multicellular organisms such as glucocorticoids (*26*) and GAGA factor (*27*) cause chromatin structure conversion under certain conditions, in this study, neither Pit-1 nor ER was a factor for conversion of the structure of PRL gene chromatin to a DNase I-hypersensitive state.

Since the positions of DHS in PRL-producing 235 cells coincide well with those of Pit-1 binding sites and ERE at the PRL gene locus (*4, 13*), PRL gene chromatin in GC cells may be in a condensed state, thus preventing the approach of Pit-1 and ER. A locus control region (LCR) is defined as a region that allows cell type-specific expression of the introduced gene in a copy number-dependent but position-independent manner in transgenic mice and stable transformants (*11, 12*). Human $\beta$-globin LCR coincides with the region having cell type-specific DHS (*11*). In the case of the human CD2 gene (*28*), LCR activity was shown again to coincide with the DHS region, although this would not be the case for the chick lysozyme gene (*29*). Thus, putative PRL LCR is likely to coincide with the DHS region. Based on the results of functional analysis of the PRL upstream region in transgenic mice, LCR activity appears to be present in the region from the transcription start site to a 3 kb upstream region of the PRL gene (*30*). No LCR-like activity was found in the PRL-P region including proximal DHS I. Putative PRL LCR should thus be present mostly in the distal DHS II (−1.6 kb) region, assuming PRL LCR corresponds to the DHS region. This region includes four Pit-1 binding sites and one ERE (*4, 31*). Pit-1 and ER are unable to convert the PRL chromatin structure to a DNase I-hypersensitive state, and thus neither Pit-1 nor ER would be the means of induction of the DNase I-hypersensitive state of PRL chromatin in 235 cells. The substance responsible would likely interact with the distal DHS region. The nucleotide sequence corresponding to PRL DHS regions has been determined (*21*). Although *in vitro* footprint analysis has revealed the positions of Pit-1 binding sites and ERE, no other *cis*-element specific for the PRL gene has so far been identified. Since *in vivo* footprint analysis corresponding to DHS studies has never been carried out, novel *cis*-elements corresponding to LCR activity may be revealed by such analysis.

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