In Vivo Genomic Footprinting of Thyroid Hormone-Responsive Genes in Pituitary Tumor Cell Lines

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We studied the effects of thyroid hormone (T_3) on nuclear protein-DNA interactions by using dimethyl sulfate (DMS) and DNase I ligation-mediated PCR footprinting. We examined an endogenous gene the growth hormone (GH) gene, and a stably transfected plasmid containing the chicken lysozyme silencer (F2) T_3 response element (TRE) gene, F2-TRE-TK-CAT, both in pituitary tumor (GC) cells. The 235-1 cell line, which expresses prolactin (PRL) and Pit-1, but not the T_3 receptor (TR) or GH, was used as a control. DMS and DNase I footprinting identified protected G residues in the Pit-1, Sp1, and Zn-15 binding sites of the GH gene in GC, but not in 235-1, cells. There was no specific protection of the tripartite GH TRE at -180 bp against either DMS or DNase I in the absence or presence of T_3 in either cell line. However, T_3 increased protection of the Pit-1 and Sp1 binding sites against DMS in GC cells. In GC cells stably transfected with a plasmid containing F2-TRE-TK-CAT or TR α , chloramphenicol acetyltransferase expression was T_3 inducible and DMS footprinting revealed both F2 TRE TR-binding half sites in a pattern suggesting the binding of TR homodimers before and during T_3 exposure. We conclude that the GH gene is accessible to specific nuclear proteins in GC, but not in 235-1, cells and that T_3 enhances this interaction, although there is no evidence of TR binding to the low-affinity rat GH TRE. The presence of TR binding to the high-affinity F2 TRE before and during T_3 exposure suggests that reversible interaction of T_3 with DNA-bound TRs, rather than transient T_3 -TR contact with TREs, determines the level of T_3 -stimulated transcriptional activation.

The expression of the rat growth hormone (rGH) gene in somatotrophs has been intensively studied to understand cell type-specific transcriptional control (3, 5, 10, 11, 43, 63). The proximal rGH promoter regulates rGH gene expression and contains DNA-binding sequences for both general and cell type-specific transcription factors, including Sp1 (at nucleotides -34 to -39), a pituitary-specific POU domain protein referred to as Pit-1, GHF-1, or GC-1 (herein termed Pit-1) at nucleotides -73 to -88 and -116 to -132 and Zn-15 (at nucleotides -95 to -110) (4, 21, 22, 24, 26, 32, 34, 57, 60, 64, 66). Pit-1 expression is required to transactivate rGH (33, 48, 62), although it is not sufficient to control cell-specific expression since it is also present in thyrotrophs and lactotrophs (15, 58). For example, the rat lactotroph cell line 235-1 expresses both prolactin and Pit-1 but little, if any, GH (53). These results indicate that additional mechanisms are required to permit rGH expression.

Thyroid hormone, 3,5,3'-triiodothyronine (T_3), is also required for normal GH expression in rat pituitary and somatotroph tumor cell lines such as GH or GC (7, 14, 21, 39, 69). The proximal rGH promoter contains a complex positive T_3 response element (TRE), consisting of three hexamer binding domains (A, B, and C) about 180 bp 5' to the start site (6, 8, 21, 26, 44, 70). Mutational studies of this positive TRE have shown that the binding affinity of the TRE half sites for the T_3 receptor (TR) homodimer correlates with TRE potency (6). However, since T_3 causes in vitro dissociation of T_3 -TR homodimers from TREs, it has been speculated that the T_3 -TR complex remains bound to DNA as a heterodimer with a retinoid X receptor or other DNA and TR cobinding nuclear proteins (9, 31, 42, 66, 67, 71, 72, 75). Recent studies have

identified a TR binding nuclear protein, the nuclear receptor corepressor, which represses TR function; T_3 releases the nuclear receptor corepressor from the TR TRE, possibly permitting interaction of the retinoid X receptor-TR complex with transcription factor IIB (12, 23). All of the currently proposed mechanisms for T_3 -activated gene transcription require persistent contact of TR with DNA. While this is easily demonstrated in vitro by gel mobility shift assays, there is no evidence of TR binding to the TRE of the GH gene or any other gene in vivo (16).

Pit-1 and T_3 -TR can cooperate to produce synergistic rGH gene activation (36, 56, 70). Transgenic experiments have shown that inclusion of the TRE at -180 in the transgene markedly enhances somatotroph-specific rGH expression (35, 36), suggesting that positive interactions between TR and Pit-1 occur (56). However, there is no in vitro evidence that TR interacts with Pit-1. On the contrary, T_3 -stimulated in vitro rGH gene transcription can occur independently of Pit-1 (59). This suggests that DNA-bound T_3 -TR and Pit-1 are independently involved in the interaction with the initiation complex to enhance transcription. In this context, it is of interest that 235-1 lactotroph cells express only low levels of TR, raising the possibility that in vivo GH expression requires T_3 -TR complexes (18).

In vivo footprinting by ligation-mediated PCR (LMPCR) has been used to indicate the presence of DNA-bound transacting factors in vivo (41). Dimethyl sulfate (DMS) can methylate guanines, making them susceptible to piperidine cleavage unless they are protected by tightly bound proteins or nucleosomes. Differences in sensitivity to purine methylation thus reflect the interactions of DNA with binding proteins. This technique has been used to study the putative interactions of ligand-binding receptors with hormone response elements, including the glucocorticoid receptor (52, 54), the androgen receptor (55), the retinoic acid receptor (RAR) (13), and TR

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(16). With the exception of the recent results obtained with RAR, no protection of the putative receptor hormone response element-DNA sequences against DMS methylation has been reported. Rather, changes in the accessibility of DNA to other DNA binding proteins was enhanced after ligand exposure, suggesting that transient ligand-receptor interactions enhanced the accessibility of these genes to specific nuclear proteins. DNase I is a relatively large molecule which can hydrolyze phosphodiester bonds in the minor groove, particularly at regions where the DNA is bent, but not if the DNA is shielded by bound proteins or is part of a tightly compacted chromatin complex. DNase I sensitivity increases in association with transcriptional activation, as has been shown for the rGH gene after exposure of pituitary cells to T_3 (45).

In the present study, we employed both DMS and DNase I genomic footprinting techniques to analyze *in vivo* protein-DNA interaction with the proximal portion of the rGH gene in GC and 235-1 cells before and after T_3 exposure. Protection of G residues in the Sp1, Pit-1, and Zn-15 binding sites on the sense strand in GC cells against DMS methylation was found in GC, but not in 235-1, cells. This indicates that the GH gene of 235-1 cells is not accessible to GH gene-specific binding proteins. No protection of the upstream TRE of the GH gene in GC or 235-1 cells was found before or after T_3 exposure, even though T_3 enhanced protection of the GH gene in GC cells. This suggests that interaction of TR with the rGH TRE is either too transient or too loose to be detected by these techniques, despite their suitability for detection of the binding of other nuclear proteins to the same gene.

To test this hypothesis, we stably transfected GC cells with a thymidine kinase (TK)-chloramphenicol acetyltransferase (CAT) plasmid with the high-affinity TRE from the chicken lysozyme silencer (F2) (2, 46, 67, 73) inserted 5' to the TK promoter together with a mouse TR α -expressing plasmid and a plasmid expressing the neomycin resistance gene. A clone, GC-8, was identified in which CAT expression was induced two- to threefold by T_3 . In these cells, there was protection of G residues of both the 5' and 3' half sites of the F2 TRE in a pattern suggesting persistent *in vivo* binding of TR homodimers, even after T_3 exposure for 6 h. These results are the first to demonstrate TR-TRE binding *in vivo*. They suggest that there is persistent contact of TR with the TRE during T_3 -induced stimulation of transcription.

MATERIALS AND METHODS

Cell culture. GC and 235-1 cells were cultured Ham's F10 medium containing 14 mM sodium bicarbonate (pH 7.4) and 2 mM L-glutamine supplemented with 10% fetal bovine serum (Gibco, Grand Island, N.Y.) until the cells grew to about 70 to 80% confluency (29). 235-1 cells were the kind gift of H. H. Samuels. For stimulation with T_3 , the culture medium was removed from the cells, they were rinsed with phosphate-buffered saline (PBS) once, and Ham's F10 medium containing charcoal-stripped 10% fetal bovine serum was added to the cells. The medium was changed after 1 day, and the incubation was continued for another 2 days. T_3 (100 nM) was diluted in Ham's F10 medium-charcoal-stripped 10% fetal bovine serum and added to cells for various times.

Transformation of GC cells. Electroporation of GC cells was performed by the method described by Forman et al. (17) with a minor modification with a Gene Pulser apparatus from BioRad Inc. GC cells were cultured in Ham's F10 medium containing 10% fetal bovine serum until the cells were 70 to 80% confluent. The cells were detached by incubation in EDTA buffer for 5 min at room temperature and washed with PBS for 5 min twice. Ten million cells were mixed with 0.4 ml of a plasmid mixture containing 10 μ g of pUTKAT3/TK108, 2 μ g of CDM13, and 0.5 μ g of pcDNA3 (Invitrogen Corp.) in PBS. pUTKAT3/TK108 contained F2 5' to the TK promoter in a CAT reporter plasmid (73). CDM13 expresses mouse TR α (51), and pcDNA3 contains the neomycin resistance gene for selection of G418-resistant, stable cell lines. The mixture was transferred into the electroporation cuvette (Gene Pulser cuvette; BioRad Inc.) and placed on ice for 10 min. Subsequently, electroporation was performed with a Gene Pulser apparatus (BioRad Inc.) at 800 V and 25 μ F with a time constant of 1.0. After electroporation, the Gene Pulser cuvette was placed on ice for 10 min. Cells were plated

onto culture dishes and incubated overnight. Starting the next day, cells were incubated in the presence of 0.6 mg of geneticin (G418) per ml to select neomycin-resistant cells. Single colonies were isolated, recultured, expanded, and screened for T_3 -responsive CAT expression. One colony, GC-8, was selected by screening for T_3 -responsive CAT expression. Southern blotting with F2 TRE-CAT as the standard indicated that there were 10 to 50 copies of F2 TRE-TK-CAT integrated per cell according to the calculation with the assumption that the size of the genomic DNA is 3×10^9 bp.

CAT assay. GC-8 cells were cultured in 60-mm-diameter culture dishes in the absence of T_3 or L-thyroxine (T_4) for 3 days, and then cells were exposed to 100 nM T_3 for 24 h at 37°C. The culture medium was removed, and cells were rinsed with PBS. Cells were treated with 200 μ l of lysis buffer (0.25 M Tris [pH 7.8], 0.5% Triton X-100) and transferred to Eppendorf tubes. The lysis mixture was vortexed for 20 s and centrifuged at $12,000 \times g$ for 20 s, and the supernatant was transferred to new tubes. The tubes were incubated at 65°C for 15 min and centrifuged for 20 s, and supernatant was transferred to new tubes again. A 100- μ l volume of supernatant was mixed with 5 μ l of *n*-butyryl coenzyme A (5 mg/ml) and 20 μ l of [3 H] chloramphenicol (0.2 μ Ci/20 μ l) and incubated at 37°C for 24 h. A 300- μ l volume of xylene was added to each reaction tube, the contents were vortexed, and centrifuged, and the bottom aqueous phase was removed. This step was repeated twice with addition of 100 μ l of 0.25 M Tris (pH 7.8). A 200- μ l volume of the xylene layer from each tube was transferred to a vial containing 3 ml of scintillation cocktail, and the radioactivity of each tube was measured. The results reported here are counts of chloramphenicol butyrate per 24 h per 60-mm dish and are averages of triplicate experiments.

***In vivo* DMS footprinting.** *In vivo* DMS methylation of DNS was performed as previously described (1, 41). After exposure to T_3 for various times, culture medium was removed from cells and 3 ml of 0.05% DMS in culture medium prewarmed at 37°C was added to cells, incubated at 37°C for 2 min, and then removed quickly. To remove residual DMS, cells were rinsed briefly three times with 3 ml of PBS and then three times more with a 30-s incubation for each exposure. After the final PBS wash, 1 ml of a lysis mixture (300 mM NaCl, 50 mM Tris · Cl [pH 8], 25 mM EDTA [pH 8], 0.2% sodium dodecyl sulfate, 0.2 mg of proteinase K per ml) was applied and partially lysed cells were collected in a tube and incubated for 4 h at 37°C. Chromosomal DNA was isolated by extraction with phenol (twice), phenol-chloroform-isoamyl alcohol (twice), chloroform-isoamyl alcohol (once), chloroform (once), and ethyl ether (once) with gentle mixing by inversion. DNA was precipitated by adding an equal volume of isopropanol, and the precipitate was transferred to 0.5 ml of Tris-EDTA buffer (TE) in a new tube, redissolved for several hours, and reprecipitated with ethanol. The precipitate was redissolved in 0.2 ml of TE, and the DNA concentration was measured by determining the optical density at 260 nm. For the *in vitro*-methylated controls, about 100 μ g of DNA was modified with 0.5% DMS and precipitated with DMS stop solution on dry ice. At the same time, about 100 μ g of DNA modified with DMS *in vivo* was also precipitated with DMS stop solution. The precipitated DNA was dissolved in 0.2 ml of 10% piperidine, and the mixture was incubated for 30 min at 90°C. The reaction product was frozen on dry ice and then dried. DNA fragments were dissolved in TE, precipitated with ethanol twice, dissolved in 30 μ l of double-distilled H₂O, and dried again. DNA fragments were dissolved in TE to 0.4 mg/ml.

***In vivo* DNase I footprinting.** The DNase I digestion reaction was performed as described by Rigaud et al. (54). Culture medium was removed from a 10-cm diameter dish, cells were rinsed with PBS, 2 ml of 0.25% trypsin was added to the dish, and the mixture was incubated for 3 to 5 min at 37°C. Cells were detached by trituration with 10 ml of ice-cold PBS, transferred to a 15-ml tube, and spun at 1,200 rpm in an AccuSpin FR centrifuge (Beckman) for 5 min at 4°C, and the supernatant was removed. Cells were washed with ice-cold PBS-1 mM phenylmethylsulfonyl fluoride once and then with physiological buffer (11 mM potassium phosphate buffer [pH 7.4], 108 mM KCl, 22 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol) once and resuspended in 0.1 ml of physiological buffer at 4°C. A 0.1-ml volume of a mixture of 0.4 mg of DNase I per ml and 0.4% Nonidet P-40 was mixed with 0.1 ml of cells in physiological buffer. This mixture was incubated for 3 min at 4°C. Subsequently, 1.6 ml of lysis solution (0.1 M EDTA [pH 8], 1% sodium dodecyl sulfate; 0.4 mg of proteinase K per ml) prewarmed at 55°C was added to the mixture, which was incubated at 55°C. After a 2-h incubation, 0.1 ml of a 5-mg/ml proteinase K solution was added to the cells and the mixture was incubated for another 2 h. DNA was isolated by phenol extraction as described above. DNA was precipitated with the same volume of isopropanol, incubated for 2 h at -20°C, and microcentrifuged for 15 min at 4°C, and the supernatant was removed. The precipitate was redissolved in 0.3 ml of TE and reprecipitated with ethanol. The precipitate was redissolved in TE to 0.4 mg/ml.

Primer design. For sense strand amplification near the transcription site, primer 1 was 5'-TCTGTCCACAGGACCTGAGCA3', primer 2 was 5'-AATCTGGGCTCGAGGGTGCTGGACT3', and primer 3 was 5'-GGGCTCGAGGGTGCTGGACT3'. For the 5' LMPER near the TRE region, primer 1 was 5'-CTTTGTGTCCAAACCACACCA3', primer 2 was 5'-CCTGCAGCCATCGC CACTCATGTAT3', and primer 3 was 5'-AGCTCTCCACACATCGCGTC CACT3'.

For antisense strand amplification near the transcription initiation site, primer 1 was 5'-TCGTCCAGTGAACAAACGATGG3', primer 2 was 5'-TGGTACC CTGCCAGATATCTCTACC3', and primer 3 was 5'-CCGTGCCAGAGTATC CTACCCCTGG3'. For the 3' LMPER near the Pit-1 binding site, primers 1 and

2 were the same as the above two primers and primer 3 was 5'GCCCATAAA CCTGGCAAAGGCGGCGG3'. The long linker was 5'GCGGTGACCCGGG AGATCTGAATTC3', and the short linker was 5'GAATTCAGATC3'.

For F2, 5'CGATGCCATTGGGATATCAACGG3', 5'TGACGCTGTTA AGCGGGTCGCTGCA3', and 5'GTAAAGCGGTGCTGCAAGGGTCGC3' were used as three sense strand primers and 5'ACGACAGGTTTCCCGACTG GA3', 5'GAGTTAGTCACTCATTCGGCACCC3', and 5'AGTCACTCAT TCGGCACCCAGGC3' were used as three antisense strand primers.

LMPCR. LMPCR was performed as described by Mueller and Wold (41), with minor modifications. The first strand of the GH promoter was synthesized in a 15-ml volume by using a mixture of 1× first-strand buffer (40 mM NaCl, 10 mM Tris · Cl [pH 8.9], 5 mM MgSO₄, 0.01% gelatin), 10 nM primer 1, 0.2 mM deoxynucleoside triphosphates (dNTPs; pH 7.0), and 16.7 mU of Vent DNA polymerase per ml which was reacted at 95°C for 1 min, 60°C for 30 min, and 76°C for 10 min. The first strand synthesized was ligated to the common linker by adding 10 ml of a ligation dilution mixture (110 mM Tris · Cl [pH 7.5], 17.5 mM MgCl₂, 50 mM dithiothreitol, 125 mg of bovine serum albumin per ml) and 12.5 μl of a ligation mixture (10 mM MgCl₂, 20 mM dithiothreitol, 50 mg of bovine serum albumin per ml, 3 mM ATP, 4 mM linker, 120 mU of T4 DNA ligase per ml) and incubating it at 17°C for 16 h. Ligated DNA was precipitated by incubation with ethanol for 2 h at −20°C, washed with 70% ethanol, and dried. DNA was dissolved in 35 μl of double-distilled H₂O. A 15-μl volume of an amplification mixture (0.33 mM long linker, 0.33 mM primer 2, and 1.3 mM dNTPs [pH 7.0] in 1× amplification buffer [40 mM NaCl, 20 mM Tris · Cl at pH 8.9, 5 mM MgSO₄, 0.01% gelatin, 0.1% Triton X-100]) and 1.5 μl of diluted Vent DNA polymerase (0.85 U/ml) were added to DNA, and amplification was performed as follows: (i) 5 min of incubation at 95°C, (ii) nine cycles at 95°C for 1 min, 65°C for 2 min, and 76°C for 3 min, (iii) nine cycles of 95°C for 1 min, 65°C for 2 min, and 76°C for 4 min, and (iv) 76°C for 10 min. The amplified product was 5' end labeled with ³²P-labeled primer 3. A 2.5-ml volume of an end-label mixture (0.46 mM end-labeled primer 3, 2 mM dNTPs, [pH 7.0], 0.2 U of Vent DNA polymerase per ml in 1× amplification buffer) was added to the amplified mixture, and two rounds of PCR were performed for 1 min at 95°C, 2 min at 68°C, and 10 min at 76°C after 5 min of incubation at 95°C. DNA from the PCR product was extracted with phenol once and precipitated with ethanol at −20°C overnight. Three independent sets of *in vivo* footprints for both strands (including *in vitro* controls) were performed for each gene. After the LMPCR, the PCR product was precipitated with 70% ethanol–0.3 M sodium acetate at −20°C overnight. The pellet was dried under a spin vacuum and dissolved in 10 μl of sample loading buffer. A 1 μl volume of the ³²P-labeled product was counted, and each sample was adjusted to the same radioactivity concentration. A 4-μl volume was loaded onto a 5% sequencing gel and run at 70 W. The gel was dried and exposed to a PhosphorImager cassette from Molecular Dynamics Inc.

Analysis of *in vivo* DMS footprints. The fluorescence image was quantitated with the Image Quant Program (Molecular Dynamics) for each band of interest. The raw values were transferred to the Microsoft Excel program and processed as follows. The raw density values were arranged in columns for cell type and stimulation condition and in rows for the position of the bands containing G residues (see tables). Raw band densities were adjusted to correct for differences in labeling between lanes of the same experiments by adjusting all of the densities to those of the same index band. The densities of the bands methylated *in vivo* are expressed relative to those of the respective band in the *in vitro*-methylated DNA for the same experiment (see tables). For statistical analysis, we used the StatView program from Abacus Concepts, Inc. (Berkeley, Calif.). Comparisons of sets of density data from the three pooled experiments were done by one-way analysis of variance followed by Scheffé's test using band densities adjusted only for differences in the labeling of the index bands.

RESULTS

T₃ increases rGH mRNA in GC, but not in 235-1 cells. There was a time-dependent increase in rGH mRNA induced by exposure to 100 nM T₃ in GC cells (Fig. 1). This was significant after 2 h and continued to increase up to 24 h. In contrast, rGH was not detectable in Northern (RNA) blots of 235-1 cell mRNA and did not appear after T₃ exposure (Fig. 1).

***In vivo* DMS footprints of the GH gene differ in GC and 235-1 cells.** Significant differences between the *in vivo* footprints of the rGH gene promoter in 235-1 and GC cells were observed. Figure 2 illustrates DMS footprints of the sense (A) and antisense (B) strands for hypothyroid and T₃-treated (21 h) GC and 235-1 cells. Figure 2C and D show sense (C) and antisense (D) labeling patterns for GC and 235-1 cell DNAs before and after 6 h of exposure to T₃. The latter is representative of three such experiments which were used for the detailed analysis of band densities presented in Table 1. Comparison of GC cell chromatin methylated *in vitro* with that

FIG. 1. Effects of T₃ on rGH mRNA production in GC and 235-1 cells as assessed by Northern blotting. All cells were incubated in T₃- and T₄-free serum for 3 days before T₃ addition. (A) GC cells incubated with 100 nM T₃ for 0, 0.5, 2, 6, 12, and 24 h. (B) 235-1 or GC cells incubated with T₃ for 0, 6, and 24 h. Equivalency of mRNA loading was monitored by ethidium bromide staining of the gels before and after transfer to nylon membranes.

from 235-1 cells methylated *in vivo* showed no significant difference in the DMS sensitivity of the sense strand before or after T₃ exposure (Fig. 2A). On the other hand, in hypothyroid cells and, to a greater extent, in T₃-exposed GC cells, there was significant protection of G residues within regions of the GH gene previously shown to bind Pit-1, Zn-15, and Sp1. These results were seen on the sense strand (Fig. 2A) as significant decreases in the intensity of G residues at positions −141, −139 to −137, and −135 in the distal Pit-1/Sp1 binding site, at position −104 in the Zn-15 binding area, and at positions −86 and, possibly, −78 in the proximal Pit-1 binding site. This pattern contrasts with that of the extracted GC chromatin (lanes Vt) or that from 235-1 cells methylated *in vivo* or *in vitro*.

Since there are no binding proteins in the extracted GC chromatin, differences in DMS susceptibility between the purine residues in these lanes are due to the DNA sequences. The densities of the *in vitro*-methylated DNA thus serve as a reference for comparison, allowing identification of density differences which are independent of protein-DNA interaction. The pattern similarity between *in vitro*-methylated GC DNA and *in vivo*-methylated 235-1 cell DNA suggests that either may serve as a control for evaluation of *in vivo*-methylated GC cell DNA. This is illustrated in the results of studies of the antisense strand (Fig. 2B). Protection of G residues in the Zn-15 and Sp1 binding sites and at nucleotides −63 and −131 in the proximal and distal Pit-1 binding sites, respectively, was found for GC, but not 235-1, cell DNA. However, conclusions regarding the significance of the latter differences are complicated by the facts that the overall density of the GC-plus-T₃ lane is less than that of the GC-minus-T₃ lane and that both lanes have lower overall densities than the 235-1 cell lanes. The variations in labeling between lanes, even in the same experiment, are difficult to eliminate and emphasize the necessity for internal points of reference to allow objective comparisons of differences in band density between lanes and between experiments.

Statistical evaluation of DMS footprints. There are several potential causes of differences in band density within and between lanes in *in vivo* footprinting experiments. These include random experimental variations, differences specific to DNA-protein interactions, those due to differences in the expression of all bands in a given lane, and those reflecting differential access of DMS to guanine residues as a consequence of the

FIG. 2—Continued.

band to that of the corresponding band in the in vitro-methylated DNA was first determined. That ratio was then used to adjust the densities of all bands in the corresponding lane of the in vivo-methylated DNA. The bands used for this purpose were thus given a value of 100 (Table 1). The densities of all bands in the in vitro-methylated DNA were expressed relative to the density of this same index band, thus preserving the experimental variations in this lane as well. Standard statistical analyses were then performed to determine the significance of differences in mean band density between in vivo- and in vitro-methylated DNAs in the three pooled experiments. To emphasize these differences, mean density ($\pm$ the standard deviation) relative to that of the respective band in the in vitro-methylated DNA is shown in the tables.

G residues in Sp1, Zn-15, and Pit-1 binding sites are protected in GC, but not 235-1 cells. Table 1 shows results of the analyses of three experiments comparing 235-1 and GC cell GH footprints before and after 6 h of exposure to 100 nM T₃, a time when GH mRNA was markedly increased in GC cells (Fig. 1). None of the G residues on either strand of the proximal GH promoter were protected in 235-1 cells (Table 1 and representative gels in Fig. 2C and D), and T₃ exposure caused no changes in band density in the 235-1 cell DNA on either strand.

The densities of the bands from the GC cells are all expressed relative to those of the bands in the in vitro-methylated 235-1 cell DNA analyzed in the same experiments (Table 1). There were no differences in in vitro-methylated DNA density between 235-1 and GC cells, indicating the equivalence of the two conditions. On the other hand, there was protection of the G residues at positions -135, -137, to -139, and -141 in the Sp1 site, at -104 in the Zn-15 binding site, and at -86 and -78 in the proximal Pit-1 site of the GH gene in the hypothyroid GC cells, and T₃ exposure increased the protection levels

further on the sense strand (Table 1). Although this implies that T₃ enhances protection of these sites, the densities of bands of the minus T₃ and plus T₃ lanes were not statistically significantly different by Scheffe's analysis, except for one G residue at -104. This may be due to the relatively large variances in the minus-T₃ samples, since the mean relative densities are all lower in the plus-T₃ lane.

There was only a single protected residue on the antisense strand (-89 or -90), and no residues were hypersensitive to DMS (Table 1). Again, the mean densities are lower than those of the respective bands in the in vitro-methylated GC DNA, suggesting that the lack of significance of the differences may relate to experimental variation rather than the absence of nuclear protein binding (Table 1).

In vivo footprinting with DNase I confirms the differences in Pit-1–Sp1 binding between GC and 235-1 cells. To analyze the GH gene for the presence of significant nuclear protein binding in the absence and presence of T₃ by using an independent technique, we employed DNase I as a probe with a shorter T₃ exposure time. An experiment representative of three similar experiments is illustrated in Fig. 3. The contrast between the DNase I footprints obtained with 235-1 and GC cells is striking. On the sense strand in GC cells in the absence of T₃, there was clear protection of the proximal Pit-1 binding site and hypersensitivity at its 3' and 5' borders. Neither of these changes was seen in the 235-1 cell GH gene. Furthermore, there was also relative protection in the Sp1 site and, again, hypersensitivity 5' to this site. On the antisense strand, the pattern was duplicated, with protection of the Pit-1 and Sp1 sites and hypersensitivity especially 5' to the Sp1 site. In general, both the sense and antisense strands of the 235-1 GH gene are relatively insensitive to DNase I with hypersensitivity at random locations in the proximal portion of the gene, the most prominent of which is just 5' to the TATA box on the

TABLE 1. Analysis of in vivo footprints of the Sp1-Pit-1-Zn-15 binding sites of the GH gene in hypothyroid or T₃-treated 235-1 and GC cells^a

Strand	Nucleotide G position	% Methylation of 235-1 cell DNA by DMS			ANOVA <i>P</i> value ^b	% Methylation of GC cell DNA by DMS			<i>P</i> value determined by:		
		In vitro	-T ₃	+T ₃		In vitro	-T ₃	+T ₃	ANOVA ^b	Scheffe's test ^c	
										In vitro vs -T ₃	In vitro vs +T ₃
Sense ^d	-154-151	100	111 ± 20	107 ± 14	0.668	102 ± 12	118 ± 9	109 ± 18	0.987		
	-148	100	100	100		100	100	100			
	-146	100	97 ± 2	113 ± 10	0.020	98 ± 2	109 ± 15	106 ± 3	0.340		
	-143	100	90 ± 15	91 ± 16	0.366	94 ± 13	98 ± 11	96 ± 14	0.576		
	-141	100	105 ± 19	112 ± 15	0.677	98 ± 6	69 ± 22	51 ± 9	0.001	0.0017	0.0010
	-139	100	93 ± 6	98 ± 25	0.868	110 ± 4	57 ± 13	40 ± 4	0.001	0.0034	0.0021
	-138-137	100	111 ± 16	113 ± 12	0.897	144 ± 38	81 ± 30	62 ± 17	0.006	0.0141	0.0091
	-135	100	122 ± 8	122 ± 31	0.375	122 ± 24	74 ± 19	49 ± 7	0.000	0.0014	0.0006
	-134	100	77 ± 25	83 ± 17	0.595	90 ± 19	134 ± 63	189 ± 38	0.042	0.1384	0.0477
	(d)Pit-1										
	-113	100	101 ± 38	95 ± 27	0.897	108 ± 11	93 ± 12	85 ± 26	0.142		
	-107	100	100	100		100	100	100			
	-104	100	108 ± 29	90 ± 12	0.711	112 ± 15	91 ± 12	66 ± 9	0.001	0.0492	0.0011
	-100	100	95 ± 34	87 ± 20	0.752	100 ± 1	100 ± 20	91 ± 31	0.680		
	-98-97	100	112 ± 33	105 ± 19	0.717	103 ± 18	124 ± 22	113 ± 50	0.449		
	-91	100	91 ± 47	93 ± 30	0.651	85 ± 17	149 ± 60	157 ± 98	0.054		
	-86	100	87 ± 57	67 ± 35	0.255	108 ± 33	56 ± 11	38 ± 14	0.006	0.0320	0.0069
	-78	100	93 ± 34	79 ± 20	0.409	80 ± 24	47 ± 8	38 ± 20	0.005	0.0255	0.0052
	-73	100	117 ± 57	100 ± 44	0.860	92 ± 21	106 ± 23	94 ± 41	0.350		
	-72	100	135 ± 64	101 ± 44	0.459	111 ± 28	137 ± 47	135 ± 74	0.550		
	-65	100	130 ± 39	128 ± 30	0.448	99 ± 16	134 ± 37	100 ± 50	0.157		
	-64	100	108 ± 35	110 ± 22	0.896	101 ± 4	130 ± 39	100 ± 59	0.211		
Antisense ^e	-158	100	76 ± 15	80 ± 6	0.202	99 ± 6	84 ± 35	74 ± 16	0.636		
	-149	100	100	100		100	100	100			
	-147	100	102 ± 12	105 ± 6	0.993	91 ± 19	96 ± 17	101 ± 17	0.981		
	-131	100	113 ± 19	105 ± 8	0.869	95 ± 14	75 ± 8	75 ± 8	0.716		
	-128	100	93 ± 10	85 ± 13	0.220	80 ± 18	88 ± 34	82 ± 24	0.951		
	-119-118	100	80 ± 10	81 ± 10	0.132	97 ± 16	79 ± 2	73 ± 12	0.172		
	-115	100	86 ± 12	96 ± 9	0.134	94 ± 13	81 ± 14	70 ± 5	0.305		
	-112	100	83 ± 7	71 ± 8	0.448	100 ± 9	62 ± 36	60 ± 37	0.627		
	-110	100	92 ± 4	86 ± 6	0.711	107 ± 8	72 ± 46	62 ± 44	0.108		
	-106	100	100 ± 26	79 ± 14	0.261	92 ± 15	109 ± 22	99 ± 18	0.733		
	-102	100	171 ± 79	103 ± 28	0.147	101 ± 10	87 ± 15	65 ± 31	0.284		
	-96	100	148 ± 51	116 ± 26	0.501	94 ± 6	120 ± 12	93 ± 15	0.326		
	-94-93	100	171 ± 69	139 ± 39	0.146	103 ± 10	112 ± 16	84 ± 15	0.375		
	-90-89	100	161 ± 65	115 ± 23	0.321	102 ± 8	102 ± 15	79 ± 11	0.007	0.9868	0.013
	(p)Pit-1										

^a Cells were treated with 100 nM T₃ for 6 h. (d), distal; (p), proximal.^b ANOVA, analysis of variance for 235-1 and GC DNAs comparing normalized values.^c The values in boldface are significantly different (*P* < 0.05) from those for in vitro-methylated GC cell DNA by Scheffe's analysis.^d The broken line separates the two portions of the gels for which the densities in each lane were adjusted to those bands at -148 and -107, respectively (see Materials and Methods and Results). All of the adjusted densities are expressed as means ± standard deviations (*n* = 3) relative to those of the corresponding bands of the in vitro-methylated GH gene from 235-1 cells.^e These densities were adjusted on the basis of the -149 band and analyzed the same way as those of the sense strand.

antisense strand. These results are consistent with those of the DMS footprints, showing no evidence of specific nuclear protein binding to the GH gene in 235-1 cells.

T₃ exposure for 2.5 h had minor effects on the DNase I sensitivity of the GH gene in GC cells, even though the level of rGH mRNA had increased by that time (Fig. 1). The protection of Pit-1 and Sp1 binding sites on the sense strand was enhanced, and the sensitivity of the proximal portion of the transcribed gene on the antisense strand was reduced. These changes did not occur in 235-1 cells. These results illustrate that DNase I footprinting, like that with DMS, demonstrates in vivo binding of nuclear proteins to the GH gene in cells which can actively transcribe the GH gene. While the DNase I results illustrate that this binding does not require T₃, both the DMS and DNase I footprints appear to be enhanced during T₃ exposure.

Neither DNase I nor DMS footprinting provides evidence of protein binding to the GH TRE. DNase I sensitivity was not convincingly reduced over the TRE area on either the sense (Fig. 3A) or the antisense (Fig. 3B) strand, either without or with T₃ (2.5 h). The slight decrease in sensitivity in the A and B domains on the sense strand after T₃ exposure was not reproducible and could be due simply to decreased labeling of the entire upstream portion of the GH gene.

We also examined the TRE by DMS footprinting (Fig. 2A and B and 4A and B). Analyses of three replicate experiments comparing DMS footprints in hypothyroid and T₃-treated 235-1 and GC cells are shown in Table 2. As expected, there was no protection of residues in 235-1 cells, since they do not express TR. More importantly, in GC cells, none of the residues in the A, B, or C domain of the GH TRE were significantly protected on either strand, despite clear evidence of

FIG. 3. In vivo footprinting of the sense (A) and antisense (B) strands of the rGH promoter in GC and 235-1 cells with DNase I. Cells were deprived of T₃ for 3 days and then incubated with or without 100 nM T₃ for 2.5 h before DNase I footprinting was performed by LMPCR. Lanes G contained a G ladder formed by in vitro methylation of GC cell DNA with DMS followed by amplification with LMPCR. These gels are representative of three similar experiments. (d), distal; (p), proximal.

protection of Pit-1 and Sp1 binding sites in the same experiments.

Protection of G residues of the F2-TRE-TK-CAT plasmid stably transfected into GC cells. Since the rGH TRE has relatively low affinity for TR binding (6, 67, 68), it was not clear whether the absence of a footprint over this TRE reflected the insensitivity of the methods or indicated that TR-DNA contacts are not sufficiently strong or persistent to protect purine residues from DMS binding. To optimize the possibility of detecting protein-DNA interactions, GC cells were stably transformed with an F2-TRE-TK-CAT construct and a CDM-8 plasmid expressing mouse TR α (CDM-13). The hexameric half sites of the F2 TRE are arranged as an everted palindrome, which is the preferred conformation for homodimer binding based on a random oligonucleotide selection strategy (28). Approximately 15 clones were analyzed for T₃-responsive CAT expression after G418 selection (see Materials

and Methods). The clone expressing the highest CAT activities with the highest CAT response to T₃ (GC-8) was further analyzed. Basal CAT expression by GC-8 cells was 8,000 to 9,000 cpm of [³H]chloramphenicol converted per 24 h per 60-mm-diameter plate. This increased two- to threefold after exposure to T₃ (100 nM) for 24 h. The F2 TRE in GC-8 cells was subjected to in vivo genomic footprinting at various intervals after T₃ treatment with primers specific for the TK-CAT plasmid. Representative examples of the patterns from three experiments are shown in Fig. 5A to D, and statistical analyses are shown in Table 3. There was significant protection of the three G residues in the 3' half site (-138, -139, and -141) (diagram in Fig. 5A and C) on the sense strand at one or more of the intervals after T₃ exposure, as well as protection of the three G residues in the upstream half site (-147, -148, and -149) on the antisense strand. Scheffe's analysis did not show significant differences in band density in the footprints at dif-

FIG. 4. In vivo footprinting of the TRE region of the rGH promoter in GC and 235-1 cells before and after DMS methylation. Experiments were performed as described for Fig. 2. (A) Sense strand; (B) antisense strand.

ferent times after T_3 exposure. Thus, there was no evidence of T_3 -induced dissociation of TR from the F2 TRE over this time interval.

DISCUSSION

Absence of footprints on the rGH promoter of 235-1 cells.

One of the most striking observations in this study is the contrast between the in vivo footprints of the GH gene in GC and 235-1 cells. With DMS, there was no difference in methylation

susceptibility between the GH gene in GC cell DNA methylated in vitro and that in 235-1 cell DNA methylated in vivo (Fig. 2). With DNase I (Fig. 3), neither the areas of reduced DNase I sensitivity nor the few hypersensitive sites bore any relationship to the well-recognized patterns of pituitary nuclear protein-GH gene interactions (4, 22, 24).

The 235-1 cell line is derived from the rat pituitary tumor 7315a, not from the MtT/W5 tumor as are the GH and GC cell lines (53, 61, 65). While GC cells produce GH and little prolactin, the opposite is true of 235-1 cells. We found no GH

TABLE 2. Analysis of in vivo footprints of the TRE binding regions of the rGH promoter^a

Strand	Nucleotide G position	% Methylation of 235-1 cell DNA by DMS			ANOVA <i>P</i> value ^b	% Methylation of GC cell DNA by DMS			<i>P</i> value determined by:		
		In vitro	- T_3	+ T_3		In vitro	- T_3	+ T_3	ANOVA ^b	Scheffe's test	
Sense	-196-195	100	70 ± 7	71 ± 11	0.100	98 ± 17	81 ± 14	100 ± 4	0.434		
	-193-192	100	86 ± 15	102 ± 13	0.097	108 ± 29	108 ± 13	129 ± 11	0.188		
	-188-187	100	97 ± 11	99 ± 6	0.929	107 ± 30	95 ± 8	106 ± 28	0.819		
	-183	100	99 ± 30	84 ± 25	0.533	92 ± 28	91 ± 29	102 ± 8	0.622		
	-178-176	100	106 ± 22	102 ± 6	0.934	101 ± 24	107 ± 24	91 ± 7	0.241		
	-173	100	83 ± 17	85 ± 15	0.097	102 ± 24	85 ± 19	85 ± 4	0.280		
	-171	100	129 ± 52	141 ± 79	0.607	102 ± 33	106 ± 44	99 ± 29	0.985		
	-167	100	93 ± 48	108 ± 52	0.595	108 ± 57	102 ± 40	99 ± 38	0.917		
	-164-163	100	100	100		100	100	100			
	-161	100	99 ± 38	94 ± 35	0.682	83 ± 23	96 ± 41	97 ± 33	0.519		
Antisense	-159	100	109 ± 18	110 ± 14	0.641	108 ± 13	131 ± 27	159 ± 30	0.027	0.3404	0.0278
	-212	100	92 ± 7	87 ± 3	0.875	106 ± 12	103 ± 10	110 ± 13	0.964		
	-211	100	127 ± 4	112 ± 3	0.048	106 ± 8	97 ± 12	107 ± 5	0.508		
	-206	100	100	100		100	100	100			
	-200	100	117 ± 18	105 ± 8	0.969	116 ± 31	100 ± 24	123 ± 29	0.823		
	-197	100	140 ± 49	113 ± 36	0.205	95 ± 15	154 ± 54	193 ± 50	0.001	0.0096	0.0005
	-180	100	151 ± 85	127 ± 56	0.598	104 ± 16	127 ± 36	129 ± 39	0.212		
	-174	100	145 ± 66	116 ± 46	0.501	90 ± 1	118 ± 40	104 ± 33	0.615		
	-169-168	100	175 ± 78	140 ± 52	0.160	111 ± 11	145 ± 55	131 ± 55	0.541		
	-166	100	134 ± 53	109 ± 39	0.399	106 ± 21	130 ± 53	100 ± 25	0.301		

^a See Table 1 footnotes for details.

^b Comparison of normalized densities for GC cell DNA in vitro, without T_3 , and with T_3 by analysis of variance (ANOVA).

FIG. 5. In vivo DMS footprinting of the F2 TRE stably transfected into GC-8 clone cells in the F2-TK-CAT plasmid. A and C, sense strands; B and D, antisense strands. The diagram shows the distance of the nucleotides from the transcription initiation site of the TK-CAT genes. These results are representative of three identical experiments. 10', 10 min.

mRNA in 235-1 cells (Fig. 1). Both cell lines express Pit-1, and GC, but not 235-1, cells express TR (18, 19). While 235-1 cells produce little or no endogenous GH mRNA, there is disagreement regarding whether or not a transfected rGH promoter is active in this cell line. Early reports showed no expression from rGH-CAT constructs in 235-1 cells (18, 24), but more recent studies employing the 235-1 cell line used in the present experiments showed that the transfected rGH promoter can function in these cells (19). It is not clear why the Pit-1 expressed in 235-1 cells does not bind to the endogenous GH gene. Since Pit-1 binds to the PRL promoter in 235-1 cells, it has been speculated that the chromatin of the GH gene in 235-1 cells is in a compact form and therefore not accessible to Pit-1 (63). Our results are consistent with that hypothesis.

No specific changes were observed in DMS or DNase I sensitivity after T₃ exposure on either the sense or antisense strand of the 235-1 cell GH gene, which is consistent with low or absent expression of TR in 235-1 cells. 235-1 cells appear to have terminally differentiated into lactotrophs, losing the capacity for either GH or TR gene expression. Ontogenically, lactotrophs are derived from somatotrophs, suggesting that there exists a mechanism to extinguish GH gene expression

and activate PRL gene expression. The role of changes in TR expression during that process, if any, is not known.

Comparison of footprints on the Pit-1, Sp1, and Zn-15 binding sites on the rGH promoter between GC and 235-1 cells. Given the lack of TR expression and of Pit-1 binding to the GH gene, 235-1 cells serve as the ideal control for evaluation of basal and T₃-stimulated GH gene transcription with the complex DMS footprinting method. In the basal state, DMS protections were detected in the Sp1, Pit-1, and Zn-15 binding sites on the sense strand (Table 1 and Fig. 2 and 6). In cells exposed to T₃ for 6 h, the protection of these G residues became more intense (Fig. 2 and Table 1). While these results suggest that T₃ enhances the binding of these nuclear proteins to the GH gene, the band densities in the minus-T₃ and plus-T₃ lanes were not significantly different by Scheffe's analysis (Table 1). Furthermore, the DNase I footprints (Fig. 3A and B) demonstrated protection of the proximal and distal Pit-1 sites and showed the expected hypersensitivity at the 3' and 5' borders of those sites, even in the absence of T₃. The results suggest that T₃ exposure is not required for Pit-1 and Sp1 binding to the GH gene but may facilitate this, presumably by enhancing chromatin availability.

TABLE 3. Analysis of F2 TRE footprints from the stably F2-TK-CAT DNA-transfected GC-8 clone^a

Strand	Nucleotide G position(s)	% Methylation by DMS					<i>P</i> value determined by:				
		In vitro	With 100 nM T ₃ at:				ANOVA ^b	Scheffe's test ^c			
			0 min	10 min	2 h	6 h		In vitro vs 0 min	In vitro vs 10 min	In vitro vs 2 h	In vitro vs 6 h
Sense ^d	−173–171	100	68 ± 22	71 ± 7	93 ± 6	107 ± 17	0.5032				
	−167	100	76 ± 19	73 ± 11	85 ± 14	101 ± 24	0.6874				
	−163–160	100	68 ± 25	77 ± 16	93 ± 5	111 ± 36	0.2263				
	−151	100	59 ± 27	51 ± 20	43 ± 12	92 ± 24	0.2027				
	−144	100	99 ± 16	81 ± 11	77 ± 17	95 ± 18	0.2069				
	−141	100	70 ± 9	67 ± 9	75 ± 10	69 ± 11	0.0034	0.0143	0.0079	0.0442	0.0371
	−139–138	100	53 ± 6	44 ± 7	44 ± 6	66 ± 6	<0.0001	0.0006	0.0001	0.0001	0.0116
	−133	100	91 ± 7	97 ± 11	95 ± 14	114 ± 18	0.2920				
	−128–127	100	87 ± 14	94 ± 18	84 ± 9	100 ± 22	0.1264				
	−118	100	100	100	100	100					
	−116	100	113 ± 5	109 ± 22	104 ± 19	104 ± 4	0.8710				
	−111–110	100	78 ± 15	89 ± 8	96 ± 7	113 ± 32	0.6697				
Antisense ^e	−121	100	117 ± 19	105 ± 14	108 ± 5	147 ± 42	0.2240				
	−124–123	100	126 ± 29	101 ± 28	105 ± 25	88 ± 35	0.8369				
	−129	100	91 ± 14	70 ± 18	79 ± 9	71 ± 16	0.3141				
	−132 (A)	100	103 ± 26	69 ± 14	80 ± 19	110 ± 31	0.1420				
	−136	100	54 ± 27	60 ± 8	53 ± 18	78 ± 20	0.1651				
	−144–143 (G)	100	94 ± 18	83 ± 22	85 ± 13	93 ± 8	0.6912				
	−146	100	108 ± 13	80 ± 9	91 ± 12	82 ± 8	0.6577				
	−147	100	65 ± 8	44 ± 9	45 ± 10	52 ± 1	0.0110	0.2271	0.0276	0.0327	0.0733
	−148	100	89 ± 10	68 ± 8	66 ± 2	73 ± 6	0.0119	0.7541	0.0464	0.0342	0.2013
	−149	100	64 ± 8	57 ± 7	48 ± 7	63 ± 8	0.0019	0.0516	0.0182	0.0044	0.0090
	−152(A)	100	96 ± 30	86 ± 50	71 ± 20	90 ± 28	0.7358				
	−155(A)	100	100	100	100	100					
	−158 (G), 157 (A)	100	115 ± 21	101 ± 20	109 ± 14	97 ± 15	0.5801				

^a GC-8 cells were hypothyroid or exposed to 100 nM T₃ for various times. Values are means ± standard deviations for band densities from three independent experiments relative to those for in vitro-methylated DNA analyzed in the same experiment. Values in boldface indicate statistically significant differences.

^b Comparison of normalized values for DNA in vitro, without T₃, and with T₃ to the density of in vitro-methylated DNA by analysis of variance (ANOVA).

^c Scheffe's analysis with normalized densities.

^d Band densities were first adjusted for each lane with reference to that of the −118 band. These were then expressed relative to the densities of the respective G residues for each lane in the in vitro-methylated DNA.

^e The densities in each lane were adjusted on the basis of the density of the −155 band and then expressed relative to the density of the corresponding G residue in in vitro-methylated DNA.

While our results are consistent with those of previous in vitro DNase I protection assays of the GH gene (reviewed in reference 63), they differ somewhat from those showing no DMS protection of Pit-1 or Sp1 binding sites in the absence of T₃ and DMS hypersensitivity at −131, −119, −118, −102, −94, and −93 on the antisense strand in GC cells at a single time point (4 h) after T₃ exposure (16). We cannot explain the differences between the two studies, but our DNase I protection results (Fig. 3) confirm the T₃-independent Pit-1–Sp1 binding observed by using DMS.

It is of interest that the protections we found with DMS

FIG. 6. Summary of in vivo footprints observed over the rGH promoter region of GC cells. Sequences of the rGH gene from −140 to −41 relative to the transcription initiation site are shown. The binding sites for Sp1, Zn-15, and Pit-1 are marked. Arrowheads denote G residues protected against DMS methylation in either the presence or the absence of T₃.

cluster on the sense strand and are strongest at the −141, −135 to −139, −104, −86, and −78 intervals, suggesting that these proteins are bound to the same face of the α-helical surface (Fig. 6). Such an arrangement would induce DNA bending, accounting for the DNase I hypersensitivity of the sequences at −58 to −60 and −97 to −104. Both Sp1 binding and Pit-1 binding are required for maximal transient expression by the GH promoter (56, 60).

No specific footprints on the TRE of the rGH promoter.

Many in vitro biochemical assays show that TR binds to TREs as homodimers or as heterodimers with partners such as the retinoid X receptor, RAR, COUP-TF, or other, yet to be defined auxiliary proteins (20, 30, 37, 38). While T₃ binding causes dissociation of homodimers, retinoid X receptor-TR heterodimers or TR monomers remain bound to the TRE (20, 30). Recent studies suggest that at least a portion of T₃-induced transactivation is explained by the dissociation of a TR-bound corepressor, N-CoR, by conformational changes subsequent to T₃-TR interactions (12, 23). Whether or not T₃ interaction also favors the binding of coactivators to the TR complex remains to be shown. However, all of the proposed mechanisms for T₃-directed transcriptional activation require the persistence of DNA-bound TR-containing complexes, at least for positive TREs. Thus, it is surprising that we could not demonstrate protection of the TRE of the rGH gene by either

FIG. 7. Summary of protein binding patterns on the sense and antisense strands of the F2 TRE. Circles indicate protected residues.

DMS or DNase I footprinting, even though the same experiments clearly identified other cell-specific DNA-protein interactions.

A previous study also did not demonstrate protection against DMS in the TRE region of the GH gene in GC cells, although T₃-induced DMS hypersensitivity was found at -158, -168, and -180 on the antisense strand (16). We did not observe these changes in GC cells (Fig. 2B and D and Table 2). Since our methods are sufficiently sensitive to detect footprints of the Pit-1-Sp1 site, we speculate that the occupancy of the TRE by TR was either too short-lived or too loose to protect G residues against DMS. Accordingly, we introduced a number of methodological variations, including reducing the DMS concentration from 0.05 to 0.002%, successively changing the time of DMS exposure from 2 min to 15 s, and lowering the ambient temperature during DMS exposure to 4°C. None of these variations, alone or in combination, led to convincing evidence of protection of G residues in the GH TRE. We conclude that despite the biochemical evidence that the TR is associated with actively transcribed chromatin in pituitary and other cells (25, 27, 47), the DMS footprinting technique is not sufficiently sensitive to detect TR binding to the GH TRE.

Protection of the F2 TRE can be demonstrated in vivo in stably transfected cells. The results in Fig. 5 and Table 3 provide the first evidence of the binding of a specific protein, presumably TR, to a TRE in intact cells. The protected residues are illustrated on the sequence in Fig. 7 and encompass G residues on both the upstream and downstream half sites. Notably, the G residue (at -144) in the six-base spacer on the sense strand is not protected, indicating the specificity of the contact sites. These results suggest that T₃ does not cause global displacement of TR homo- or heterodimers from the F2 element in vivo during T₃-induced transcription of CAT expression. This, in turn, is consistent with recent evidence that the persistent binding of homodimers containing a TR mutation associated with T₃ resistance can explain the hypersensitivity of the F2 element to dominant negative inhibition (40, 49, 50, 73, 74).

Together with the GH gene footprinting results, these results suggest that persistent TR-TRE contact may occur but that this is difficult to demonstrate in vivo except on a high-affinity TRE. The GC-8 cell is not normal, having a number of randomly integrated copies of the F2-TRE-TK-CAT plasmid in the genome. However, since CAT expression is T₃-responsive, it can serve as a model system for modifying the techniques for in vivo DMS footprinting to enhance sensitivity and specificity so that genes such as GH might be restudied. The absence of changes in the F2 TRE footprints during T₃ exposure implies further that T₃-induced transcription is due to intermittent binding of T₃ to TRE-bound receptors rather than by transient T₃-TR interactions with the TRE.

Comparisons with results of other attempts to footprint HRES in vivo. In vivo footprinting of well-recognized HRES has been quite difficult. A lack of success in using DMS or DNase I to footprint the glucocorticoid receptor binding site on the TAT gene led Rigaud et al. to propose a "hit-and-run"

mechanism for glucocorticoid receptor binding to the TAT gene which made the DNA available for HNF-5 binding to its binding site (54). There is no specific event, such as HNF-5 binding, recognized to follow T₃-TR-GH gene interaction, but the enhanced chromatin availability induced by T₃ exposure may be an equivalent event allowing greater Pit-1 and Sp1 binding (Table 1 and Fig. 2 and 3). Reik et al. observed a decrease in DNase I sensitivity in the GRE of the TAT gene 1 h after glucocorticoid exposure but no major changes in DMS footprints in the same area (52). Difficulty was reported in attempts to footprint the androgen receptor element of the mouse sex-limited protein gene by using DNase I, even though binding sites for multiple other proteins could be recognized (55). This is similar to our experience with the rGH gene.

A recent report described successful footprinting of the RAR element of the RAR β 2 gene (13). In contrast to results obtained with the F2 TRE, those investigators found no binding of the RAR to the RAR element until cells were exposed to ligand. This suggests either that there are basic differences between RARs and TRs in terms of DNA binding in the absence of ligand or that the constitutive overexpression of TR α in GC-8 cells resulted in an unphysiological association of APO-TR with the F2 TRE.

In summary, we have successfully applied genomic footprinting techniques to studies of the cell type-specific expression of the GH gene and the F2 TRE. The results obtained provide an explanation for the lack of GH expression by 235-1 cells in that there was no binding of Pit-1 or Sp1 to the GH gene, implying its lack of availability for transcription. While we were unsuccessful in footprinting the TRE of rGH by either DMS or DNase I in GC cells, we did identify a footprinting pattern consistent with the in vivo binding of TR homo- or heterodimers by the F2 TRE when this was stably transfected into GC cells. The results show that complexes containing T₃-TR can remain tightly bound to this element in vivo during T₃-induced gene transcription.

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