Cell- and Sequence-specific Binding of Nuclear Proteins to 5'-Flanking DNA of the Rat Growth Hormone Gene

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Stimulation of growth hormone gene transcription in several rat pituitary cell lines (e.g. GC and GH₁) is mediated by a thyroid hormone nuclear receptor which is a DNA binding protein. We report that these cell lines contain nuclear proteins which selectively interact with sequences found within the first 236 base pairs of 5'-flanking DNA of the rat growth hormone gene. Sequences found between -104 and -49 base pairs, relative to the transcription initiation (cap) site, bind to nuclear protein(s) which appears to be cell type specific and generate a DNase I resistant footprint on both strands between -95 and -68. A distinct protein component(s) selectively binds to DNA between -236 and -146 but is not cell type specific. These regions correspond to those found in gene transfer studies to be important in mediating basal expression (-104/+7) and thyroid hormone-regulated expression (-236/-146) of the gene.

Thyroid hormone stimulates growth hormone gene expression in somatotrophic cells of the rat anterior pituitary and in several rat pituitary cell lines (1-7). This effect is mediated by a chromatin-associated receptor which is a DNA binding protein (1, 7, 8). We have presented evidence that thyroid hormone rapidly stimulates the transcription rate of the rat growth hormone gene in cultured GC cells which is proportional to the level of thyroid hormone-receptor complexes (7). Recent gene transfer studies have indicated that 5'-flanking DNA of the rat growth hormone gene contains cis-acting element(s) which mediate regulated expression by thyroid hormone (9-11). These sequences appear to be contained within the first 236 base pairs of 5'-flanking DNA of the gene (10, 11).

Although the rat growth hormone gene has been widely used to study regulated gene expression by thyroid hormone (1-10), no studies have been reported which have examined the cell- and sequence-specific binding of putative trans-acting factors with the gene. Nondenaturing polyacrylamide gel electrophoresis (12-17) was used to identify whether nuclear proteins specifically interact with 5'-flanking DNA of the gene. This procedure is based on the observation that DNA-protein complexes have a decreased electrophoretic mobility compared with the unbound DNA fragment resulting from alterations in charge density and/or DNA conformation (15). This approach has been effectively used to study the interaction of purified procaryotic regulatory proteins with specific DNA sequences (12-14). More recently this technique has been applied to impure protein preparations where a synthetic duplex alternating DNA polymer, poly(dl-dC), is added to inhibit nonspecific DNA-protein interactions (17).

In this study using gel electrophoresis we provide evidence that a protein(s) found in growth hormone-producing cells selectively binds to DNA between -104 and +7 relative to the cap site of the gene. DNase I footprinting further localized the binding to sequences between -95 and -68 base pairs which contains the "CAAT" homology (18). A separate protein(s) of lower abundance, which is not cell type specific, binds to 5'-flanking DNA between -236 and -146. These sequences are located in the regions found by gene transfer studies to be involved in mediating basal (-104/47) and regulated expression of the gene by thyroid hormone (-236/-146) (11).

**EXPERIMENTAL PROCEDURES**

Materials—[α-32P]dCTP, [γ-32P]dATP, or [γ-32P]ATP (each at 3000 Ci/mmol) were obtained from Du Pont-New England Nuclear. DNase I (ribonuclease free) was from Worthington and calf intestinal alkaline phosphatase (molecular biology grade) was from Boehringer Mannheim. All other enzymes were obtained from either New England Biolabs or Boehringer Mannheim and, unless indicated otherwise, were used under the conditions recommended by the suppliers. Duplex poly(dl-dC) was obtained from Pharmacia P-L Biochemicals. Reagents used for gel electrophoresis were obtained from Bio-Rad or Eastman.

**Cell Culture Conditions**—The growth hormone-producing rat pituitary cell lines GC and GH₁, were cultured with Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum (GIBCO) (7, 9) while GH₁ cells, a related cell line, were cultured with Ham's F-10 medium containing 1.1 mM MgCl₂ and 1.1 mM CaCl₂, 10% fetal calf serum (GIBCO) (3), H4TG rat hepatoma cells (American Type Culture Collection) and Rat2 cells, a fibroblastic-like line (19), were cultured using the same media as GC cells.

**Assay of DNA-Protein Binding by Gel Electrophoresis**—Cell nuclei were isolated (20) and extracted with buffer containing 0.4 M KCl, 20 mM Tris-HCl (pH 7.85 at 25 °C), 0.25 M sucrose, 1.1 mM MgCl₂, and 10 mM 2-mercaptoethanol (20). These conditions give the most efficient extraction of thyroid hormone nuclear receptor and the salt and buffer conditions are similar to those used to prepare nuclear extracts for in vitro transcription assays (21). The nuclear extracts were stored in small aliquots at -80 °C in 33% glycerol, and freshly thawed material was used in each experiment. A Pst digestion fragment of the cloned rat growth hormone gene (-530 to +69) (18) was cleaved with XhoI, and the fragment extending from -530 to +7 was subcloned and amplified (22). A restriction map of the fragment is shown in Fig. 2a. After cleavage by the appropriate restriction enzymes, the abbreviations used are: poly(dl-dC), alternating duplex poly(dG-dC), and poly(dA-dT).

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various fragments were isolated by gel electrophoresis and labeled by filling in the cohesive ends with the large (Klenow) fragment of DNA polymerase I using \([\alpha-^32P]dCTP\) or \([\alpha-^32P]dATP\) (22). The DNA fragment(s) (6000 dpm; about 0.06 ng or 10–12 fmol) were incubated with 2 µg of nuclear extract protein at 25 °C for 30 min in 20 µl of buffer composed of 15 mM HEPES (pH 7.5 at 25 °C), 60 mM KCl, 5 mM MgCl2, 1 mM EDTA, 5.3 mM dithiothreitol, 12% glycerol, and varying amounts of duplex poly(dI-dC). The samples were chilled to 6°C and loaded onto a 4% polyacrylamide gel (acrylamide:bisacrylamide, 29:1) which had been pre-electrophoresed for 120 min at 4 °C using buffer consisting of 6.7 mM Tris-HCl (pH 7.5 at 25 °C), 1 mM EDTA, and 3.3 mM sodium acetate (16). The electrophoresis was performed at 4 °C at a voltage gradient of about 10 V/cm with rapid buffer circulation and the electrophoresis was stopped when the bromphenol blue marker migrated to the bottom of the gel. The gel was soaked in 5% glycerol for 20 min with gentle shaking and, after air drying, was autoradiographed at -80 °C for 5–20 h using Kodak X-Omat AR film and a Du Pont Lightning Plus intensifying screen.

DNase I Footprinting Studies—The FnuDII site at -145 (Fig. 2a) was converted to a BglII site using linkers and the resultant BglII/XhoI (-145/+7) fragment was subcloned. The DNA was first cleaved at the BglII site to label the end at -145 with either the Klenow fragment and [\(\alpha-^32P\)]dCTP (noncoding strand) or T4 polynucleotide kinase and [\(\gamma-^32P\)]ATP (coding strand). The DNA was then cleaved with XhoI, and the labeled -145/+7 fragment was isolated by polyacrylamide gel electrophoresis. DNase I footprinting experiments (23) were performed using the same conditions as for gel electrophoresis except that the reaction was scaled up 10–50-fold. After 15 min at 25°C the reactions were adjusted to 10 mM Mg2+ and 2.5 mM Ca2+.

RESULTS

Gel Electrophoretic Identification of Protein-DNA Interactions Using Poly(dI-dC) as a Competitor—Fig. 1 shows the effect of GC cell nuclear extracts on the electrophoretic migration of a \(^{32}P\)-end-labeled BglII/XhoI restriction fragment which extends from -236 to +7 relative to the cap site of the rat growth hormone gene (18). When nonspecific DNA-protein interactions were inhibited by increasing amounts of poly(dI-dC) (lanes 2–7), two \(^{32}P\)DNA species were detected (lane 7) with mobilities less than the free \(^{32}P\)DNA fragment (lane 1). Proteinase K digestion (lane 8) indicates that the decrease in electrophoretic mobility reflects the interaction of protein with DNA. The more abundant (lower) protein-DNA complex (Fig. 1, lane 7) was always observed while the amount of the other (upper) complex varied among different experiments. When Escherichia coli DNA was used instead of poly(dI-dC), similar protein-DNA complexes were formed but the effective concentration range was very narrow. With 2 µg of nuclear extract protein, 1 µg of E. coli DNA gave similar protein-DNA complexes as 2–5 µg of poly(dI-dC), while 2 µg of E. coli DNA significantly decreased the amount of protein-DNA complex formed. This has been observed in studies with other genes (17) and presumably results from the fact that natural DNA contains short sequences which have sufficient homology to compete with specific DNA sequences at high competitor concentrations.

Interaction of Nuclear Proteins of GC Cells with Different 5’-Flanking Regions of the Rat Growth Hormone Gene—To further define the binding regions in the 5’-flanking DNA, we examined a series of DNA restriction fragments ranging from -530 to +7 (Fig. 2a). In these studies we identified two regions which selectively bind to distinct proteins from GC cell nuclei. For the purpose of discussion we refer to these two binding domains as regions I and II. Region I binding occurs within the 5’-flanking DNA between the AluI (−104) and the XhoI restriction site (which is cleaved between +7 and +8) to form an abundant complex which electrophoreses as a broad radiolabeled band of lower mobility than the free DNA fragment (Fig. 2b, lane 2). The TaqI/XhoI fragment (−48/+7), containing the TATA homology, showed no interaction with nuclear extract protein under the conditions used (Fig. 2b, lane 1) suggesting that sequences in the −104/+7 fragment which form the protein-DNA complex lie between −104 and −49. The FnuDII/XhoI fragment (−145/+7) (Fig. 2b, lane 3) generated the same abundant complex seen with the −104/+7 fragment and an additional species of lower mobility to give a similar pattern as the −236/+7 fragment (BglII/XhoI) (Fig. 1, lane 7).

The different electrophoretic pattern formed with the −104/+7 and −145/+7 fragments suggests that additional proteins bind to the longer fragment. However, the amount of this additional complex varied among different experiments. DNase I footprinting studies (Fig. 2c) were carried out in an experiment in which the two complexes could be identified by gel electrophoreses:’s. Only one DNase I protected region was detected which was more predominant on the noncoding strand (−68 to −95) (lanes 3 and 4) than the coding strand (−68 to −90) (lanes 5 and 6). No DNase I protected regions were detected between −104 and −145 under the conditions used to identify the DNase I footprint between −68 and −95.
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which are involved in the protein-DNA interaction (25).

**Competition Studies Using Homologous and Heterologous DNA Fragments**—Competition studies (Fig. 3) indicate that proteins which bind to region II (−236/−146) are distinct from those which interact with sequences in region I (−145/−7). When 50 ng of the unlabeled −145/+7 or −104/+7 fragment was incubated with 0.05 ng of 32P-labeled DNA from −145/+7, the formation of labeled protein-DNA complexes was completely inhibited (Fig. 3a, lanes 2 and 3). In contrast, 50 ng of the −236/−146 fragment did not inhibit protein-DNA complex formation of the labeled −145/+7 fragment (Fig. 3a, lane 4). Complex formation of labeled DNA from −104/+7 was also inhibited by the unlabeled homologous fragment but not heterologous DNA from −236/−146 (Fig. 3a, lanes 6 and 7). In like fashion, complex formation of labeled −236/−146 DNA was inhibited by the homologous fragment but not by DNA from −145/+7 (Fig. 3b). When GC cell nuclear extracts were incubated with 0.05 ng of labeled DNA from −236/+7 and 30 ng of unlabeled DNA from −145/+7, the protein-DNA complex formed migrated as a narrow labeled species characteristic of the complex formed by the

When the BglII/FnuDII fragment extending from −236 to −146 was incubated with GC cell nuclear extract, it formed a complex which migrated as a narrow rather than a broad labeled band (Fig. 2b, lane 4). We refer to DNA in the −236/−146 fragment as binding region II. An identical gel electrophoretic complex was formed using a labeled −236/−130 fragment (not illustrated). In contrast, sequences from −312 to −237 and from −530 to −313 did not generate any high affinity protein-DNA complexes (Fig. 2b, lanes 5 and 6). In vitro addition of L-T3 did not alter the results of Fig. 2b. Although we have been able to use DNase I to footprint the binding region in the −104/+7 fragment (Fig. 2c), no DNase I protected regions were identified using a −236/−130 fragment even when the complex was isolated from the gel. Gel electrophoresis has been reported to detect protein-DNA complexes which are not identified by DNase I protection experiments (25). Therefore, other techniques will be required to precisely locate the sequences in the −236/−146 region

**Fig. 2.** Selective binding of nuclear proteins in GC cell nuclear extracts to restriction fragments of the 5′-flanking region of the rat growth hormone gene. a, restriction fragments of the 5′-flanking region used in the binding experiments in b. Each reaction in b contained 2 μg of extract protein, 4000 ng of poly(dI-dC), and 5000 dpm of the respective 32P-end-labeled DNA fragment. In each lane in b the radiolabeled species with the highest mobility (lowest in each line) migrated to the same position as the [32P]DNA fragment which was not incubated with nuclear extract (not shown). In b the restriction fragments used were: lane 1, −48/+7; lane 2, −104/+7; lane 3, −145/+7; lane 4, −236/−146; lane 5, −312/−237; and lane 6, −530/−313. c, DNase I footprinting of the −145/+7 fragment labeled at the 5′-end. Details are given under “Experimental Procedures.” Lane 1, G + A sequencing ladder; lane 2, T + C sequencing ladder; lanes 3 and 4, noncoding strand incubated without (−) or with (+) nuclear extract; lanes 5 and 6, coding strand incubated without (−) or with (+) nuclear extract.

**Fig. 3.** 5′-Flanking DNA from −145/+7 (binding region I) and −236/−146 (binding region II) interact with different nuclear proteins. Three thousand dpm of each 32P-labeled DNA fragment and 3000 ng of poly(dI-dC) were used in each reaction. The [32P]DNA fragment and the excess of unlabeled DNA fragment (indicated below) were mixed well before addition of the nuclear extract. a, competition studies of labeled DNA fragments containing binding region I (lanes 1–4, −145/+7; lanes 5–7, −104/+7) with unlabeled DNA fragments containing binding region I or binding region II. In lanes 1 and 5 the [32P]DNA fragments (about 0.05 ng) were incubated with 1 μg of GC cell nuclear extract protein without any unlabeled DNA fragments. Lane 2 contained 50 ng of the unlabeled −145/+7 fragment; lanes 3 and 6 contained 50 ng of the unlabeled −104/+7 fragment; lanes 4 and 7 contained 50 ng of the unlabeled −236/−146 fragment. b, competition studies of a labeled DNA fragment containing binding region II (lanes 1–3, −236/−146). The radiolabeled fragment −236/−146 (about 0.05 ng) was incubated with 3 μg of GC cell nuclear extract protein without unlabeled DNA (lane 1), with 30 ng of the same unlabeled fragment (lane 2), or 30 ng of the −104/+7 unlabeled fragment (lane 3), c, a [32P]DNA fragment (about 0.05 ng) containing binding regions I and II (−236/−146) was incubated with 2 μg of GC nuclear extract protein in the absence of any unlabeled DNA fragment (lane 1) or with 30 ng of the unlabeled fragment from −145/+7 (lane 2) which contains only binding region I.
producing rat pituitary cell line, generated a pattern similar with the -236/-146 fragment (Fig. 4c). More of the complex proteins from Rat2 and H4TG cells (Fig. 4b). In contrast with the rat hepatoma H4TG and the Rat2 fibroblastic cell lines to GC and GH, cells (not shown). In contrast, extracts from the rat hepatoma H4TG and the Rat2 fibroblastic cell lines did not give the same pattern of binding. Instead, nuclear proteins from the H4TG and the Rat2 lines generated a complex having a very low electrophoretic mobility seen near the top of the gel. Gel electrophoretic shift studies were also carried out with nuclear extracts from rat liver and testes (not shown). These extracts, when incubated with the -145/+7 fragment, gave results identical to that observed with nuclear proteins from Rat2 and H4TG cells (Fig. 4b). In contrast with the -145/+7 fragment, nuclear proteins from each of the rat cell lines formed the same high affinity protein-DNA complex with the -236/-146 fragment (Fig. 4c). More of the complex appeared to be formed by nuclear extracts of GC and GH1, than Rat2 and H4TG cells. Nuclear extracts from rat liver and testes also generated the same gel shift band with the -236/-146 fragment which was about 50% less than that observed with extracts from GC cells (not shown).

### DISCUSSION

Sequences which form protein-DNA complexes in the -104/+7 fragment appear to be localized between -104 and -49 (Fig. 2b) and generate a DNase I footprint between -68 and -95 which spans the CAAT homology found at about -80 (18). This gel electrophoretic complex was only identified in cells which express the rat growth hormone gene (Fig. 4, a and b), suggesting that sequences in this region recognize cell-specific proteins which mediate basal and/or cell-specific expression of the gene. This notion is supported by transient expression experiments which indicate that basal expression in GC and GH, cells is dependent on sequences located between -104 and +7 and that no expression occurred in Rat2 or H4TG cells (11). When sequences between -236 and -146 were deleted, regulated expression by thyroid hormone was eliminated (11). This suggests that proteins which bind to sequences from -236 to -146 may play a role in mediating regulated expression by hormone. Protein(s) which bind to this region were found in each of the rat cell lines examined but were less abundant in the Rat2 and H4TG cells (Fig. 4c) which have about 60–75% less thyroid hormone receptor than the somatotrophic cell lines.2

The amount of this protein in GC cells is much less abundant than the protein(s) which bind to DNA from -104 to +7 and is in the same range as the thyroid hormone nuclear receptor. The amount of the protein which binds to the -236/-146 fragment can be estimated from the gel electrophoretic assays. Using 10 fmol of labeled fragment, about 20% of the DNA forms a protein-DNA complex (Fig. 2b, lane 4). Increasing the amount of the fragment does not substantially increase the amount of complex, suggesting that there is about 2 fmol of binding protein/2 μg of GC cell nuclear extract protein. This amount is in the same range as the amount of receptor extracted from GC and GH1 nuclei by 0.4 M KCl (100 fmol/100 μg nuclear extract protein) (7, 20). The observation that rat liver nuclear extracts generate about 50% of the protein-DNA complex with the -236/-146 fragment is in keeping with the relative difference in abundance of receptor in GC cells and rat liver (7, 9, 20). However, nuclear extracts from rat testes also generated the same gel shift pattern with the -236/-146 fragment which was about 50% of that found with GC cell extracts. Studies of receptor abundance in various rat tissues by in vivo injection of L-[3H]T3 suggest that nuclei from testes contain only 0.4% of the receptor of rat liver (26). In vitro L-[3H]T3 binding studies using testes nuclear extracts, however, indicated a receptor abundance which was about 10% of that found in GC cell extracts.2 The differences noted in the in vitro3 and the in vivo abundance of receptor (26) in the testes may be secondary to low levels of perfusion of the testicular cells which contain receptor or to a decrease in cell entry of L-[3H]T3.

The discrepancy between receptor abundance and the amount of protein-DNA complex formed with testicular nuclear extract and the -236/-146 fragment does not permit us to conclude that the protein(s) which binds to the fragment is receptor. Our results indicate, however, that a wide variety of rat cell lines contain a nuclear protein(s) which binds in a sequence-specific manner to the region of the rat growth hormone gene which is involved in mediating regulated expression by thyroid hormone (11). Recent studies with chick and human cells indicate that one of the cellular homologues of the avian erythroblastosis virus v-erb-A gene encodes a thyroid hormone receptor (27, 28). Several other distinct cellular homologues of the v-erb-A gene have been cloned (29–31) which, like the 75-kDa viral gag-erb-A gene product (27), may retain the DNA binding domain but may not bind thyroid hormone. Whether the protein(s) which can interact with the -236/-146 fragment represents receptor as well as related erb-A protein homologues which do not bind hormone is under investigation.

Our results suggest, however, that the unique expression of

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2 H. H. Samuels, J. Casanova, and Z.-S. Ye, unpublished observation.
the growth hormone gene in somatotrophic cells may be determined by cell-specific regulatory factors which bind to sequences found within the first 104 base pairs of 5'-flanking DNA of the gene. The functional role of this cell-specific protein(s) and the protein(s) which interact with the region determined by cell-specific regulatory factors which bind to DNA sequences found within the first gene.

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