Resolution by Diagonal Gel Mobility Shift Assays of Multisubunit Complexes Binding to a Functionally Important Element of the Rat Growth Hormone Gene Promoter*

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The transcription of a given gene is dependent upon the presence and availability (Becker et al., 1987; Baeuerle and Baltimore, 1988) of both \( \alpha \)-elements and trans-acting factors representing the undissociated complexes which lie on a diagonal line. Two of the five complexes also contain an additional subunit in common whereas two other complexes appear to contain completely different subunits interacting with the common DNA-binding unit. All five complexes copurify during GHF3-binding site-specific DNA affinity chromatography, and this fraction stimulates \textit{in vitro} transcription in a GHF3-binding site-dependent fashion. Thus, a functionally important region of the rat growth hormone gene promoter interacts with a DNA-binding transcription factor which in turn acts as a docking site for other proteins.

DNase I footprinting identifies a tissue-general factor, GHF3, binding to the rat growth hormone promoter between nucleotides \(-239\) and \(-219\). Mutation of the GHF3-binding site reduces promoter activity to 30% of that of the wild-type promoter after transfection into GC cells. Southwestern blotting and protein/DNA cross-linking experiments demonstrate that the GHF3-binding factor migrates as a 43-kDa protein. However, multiple GHF3 factor/DNA complexes with different electrophoretic mobilities are detected by gel retardation analysis. A novel technique, the diagonal gel mobility shift assay, is used to demonstrate that five of the different complexes represent multisubunit structures containing a common DNA-binding subunit. In this method, the multisubunit complexes resolved by one-dimensional gel mobility shift assays are observed to partially dissociate during electrophoresis in a second dimension with the DNA-binding subunit detected as a common signal directly below those signals representing the undissociated complexes which lie on a diagonal line. Two of the five complexes also contain an additional subunit in common whereas two other complexes appear to contain completely different subunits interacting with the common DNA-binding unit. All five complexes copurify during GHF3-binding site-specific DNA affinity chromatography, and this fraction stimulates \textit{in vitro} transcription in a GHF3-binding site-dependent fashion. Thus, a functionally important region of the rat growth hormone gene promoter interacts with a DNA-binding transcription factor which in turn acts as a docking site for other proteins.

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Materials and Methods

Extract Preparation—Preparation and extraction of rat liver nuclei was performed essentially as described in Gorski et al. (1986) with the further addition of the proteinase inhibitors, antipain, leupeptin, chymostatin, and pepstatin A (Sigma) at final concentrations of 5 \( \mu \)g/ml each during the nuclei preparation and extraction steps. GC cell nuclei were similarly prepared except the cells were initially homogenized in 0.25 M sucrose-containing buffer and the "homogenization buffer" contained 1.0 M sucrose in the subsequent steps. Following homogenization, the nuclei were purified over two sucrose cushions, the chromatin was washed with 0.36 M (NH\(_4\))\_2SO\(_4\), and the extract concentrated by (NH\(_4\))\_2SO\(_4\) precipitation as described by Gorski et al. (1986). Extracts were resuspended in 25 mM Hepes, pH 7.9 (21 °C), 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol. Some extracts were prepared using a 0.64 M (NH\(_4\))\_2SO\(_4\) wash but did not appreciably affect \textit{in vitro} transcription or gel mobility shift assays (data not shown).

DNA Binding Assays—DNase I footprints were done by incubating protein with end-labeled rGH promoter fragments (in all experiments shown, fragments are end-labeled using T\(_{4}\) polynucleotide kinase at the sites indicated below), then treating the complexes formed with 50 ng of DNase I ( Worthington) for 1 min (purified fractions or nuclease A) or 200 ng of DNase I for 3 min (80 \( \mu \)g crude extract) at room temperature. DNase I digestion was stopped with 2.5 volumes of 4 M NH\(_4\)\_2CO\(_3\) 0.1 M EDTA, precipitated, and loaded onto a sequencing gel. For the gel mobility shift assays, 1 \( \mu \)l (6 \( \mu \)g) of extract was incubated at room temperature for 20 min in a 20-\( \mu \)l final volume with (i) 1 \( \mu \)l (~5fmol) of radiolabeled DNA in 100 mM KCl, (ii) 1 \( \mu \)l

The abbreviations used are: rGH, rat growth hormone; Hepes, 4-
(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; Pipes, 1,4-piperazinediethanesulfonic acid.
Two-dimensional Gel Shift Analysis of Multisubunit Transcription Factors

(4 μg) of poly(dI-dC) (Pharmacia LKB Biotechnology Inc.), (iii) 8 μl of 30 mM Hepes, pH 7.6 (0 °C), 120 mM KCl, 10 mM MgCl2, 2 mM EDTA, and 24% glycerol. For the competition studies, each 1 μl (500 ng) of the indicated, unlabeled oligonucleotides in 100 mM KCl was substituted for 1 μl of 100 mM KCl added to the unincubated incubations. Following incubation, the samples were put on ice and loaded directly onto pre-run (2 h) 4%, 29:1 (poly-bis-acrylamide) gels pre-cooled to 4 °C. Electrophoresis was buffered at 0.7 M Tris, 1 mM EDTA, 3.3 mM CH3COONa, and adjusted to pH 7.5 (21 °C) with CH3COOH.

DNase I footprints of gel-shifted material were performed by digesting the complexes after 20 min of incubation with 50 ng of DNase I (Worthington) at room temperature for 1 min (stopped by the addition of EDTA to 12.5 mM) before loading onto the native polyacrylamide gel.

Clone Construction and Fragment Labeling—Plasmid 237/148 was constructed by inserting a rGH promoter fragment from BglII (-237) to Thal (-148) into the BglII/SmaI-cleaved pUC18 derivative, pUC18BA (see Schirm et al., 1987). The 87-bp Hinfl (-285)/Fnu4HI (-198) rGH promoter fragment was blunt-ended with Klenow polymerase before insertion into the SmaI site of pUC18BA to generate the clone -285/-198. Fragments were labeled in the poly linker at either the EcoRI or the HindIII sites for footprinting (Figs. 1 and 4) or gel mobility shift assays (Figs. 3 and 8). The -230 to -191 mutant series of clones (Fig. 2) was constructed by M13 mutagenesis using appropriate oligonucleotides and verified by sequencing.

Cross-linking of Protein-DNA Complexes—Gel mobility shift experiments were done using GC extracts and the 25-bp GHF3 oligonucleotide 1 (see Fig. 5A) labeled with T4 polynucleotide kinase. Complexes of differing electrophoretic mobilities were isolated, and these acrylamide plugs were UV irradiated (254 nm, UVP Inc., San Gabriel CA) for 10 min on ice at a distance of 9 mm (Petri dish lid). Samples were eluted overnight into Laemmli gel loading buffer and fractionated by SDS-PAGE (Laemmli, 1977) the next day.

DNA Transfection—10 μg of rGHCAT and 2 μg of RSVCAT were transfected into GC cells grown as described in West et al. (1987). RNase protection experiments were performed as follows: 30 μg of RNAs from each transfected or 25% of each RNA generated by in vitro transcription were hybridized overnight at 60 °C to 80% HCONHZ, 400 mM NaCl, 40 mM Pipes, pH 6.7, 1 mM EDTA with probes complementary to the indicated transcripts. Each hybrid was then digested at 37 °C for 60 min with 500 μl of λ RNase A in 0.3 M NaCl, proteinase K digested, phenol extracted, precipitated, and loaded onto 6% sequencing gels (Figs. 2 and 7). Probes detect specific protein/DNA interactions by DNase I footprinting (Fig. 5A). DNase I digestion after labeling the fragment on the antisense or sense strand, respectively, and incubating with 80 μg of nuclear extracts prepared from either the rGH-producing GC cell line (lanes 3 and 7), rat liver (lanes 4 and 8), or a non-rGH-producing rat pituitary cell line, 235-1 (not shown) (compare with naked DNA control, lanes 2 and 6). Thus, a nuclear factor, henceforward referred to as GHF3, footprint a site within the rGH promoter (Fig. 1B) and is present in cells not expressing growth hormone, as well as cells in which rGH is expressed.

In order to determine if GHF3 binding correlates with promoter activity, six separate five to seven base pair mutations (Fig. 2B) spanning positions -230 to -191 (named according to the inclusive boundaries of the mutated nucleotides) were analyzed for their effects on the activity of the rGH promoter (Fig. 2A): the wild-type or mutant rGH promoters, linked to the bacterial chloramphenicol acetyltransferase (CAT) gene, were cotransfected with a construct containing the Rous sarcoma virus (R5V) promoter also linked to the CAT gene. Signals detected with a labeled RNA probe complementary to the rGHCAT constructs corresponded to transcripts properly initiated from the rGH transcription start site (rGH, Fig. 2A). RSVCAT transcripts were detected by virtue of their homology to the CAT portions of the probe and served as internal controls for DNA transfection, RNA recovery and RNA mapping. Only the -230,

RESULTS

Mutation of the GHF3-binding Site Decreases Promoter Activity—A cis-acting promoter element contributing to rGH expression between nucleotides -195 and -237 was previously identified by 5′ serial deletions of the rGH promoter (Nelson et al., 1986; West et al., 1987; +1 = transcription start site); correctly initiated transcripts are produced only 30% as efficiently from a clone containing rGH promoter sequences up to position -195 as compared with the promoter containing sequences up to position -237 (data not shown). Fragments spanning this region (from -198 to -285) were analyzed for specific protein/DNA interactions by DNase I footprinting (Fig. 1A). Regions of the rGH promoter between -222 and -230 or between 210 and 237 were protected against DNase I digestion after labeling the fragment on the antisense or sense strand, respectively, and incubating with 80 μg of nuclear extracts prepared from either the rGH-producing GC cell line (lanes 3 and 7), rat liver (lanes 4 and 8), or a non-rGH-producing rat pituitary cell line, 235-1 (not shown) (compare with naked DNA control, lanes 2 and 6). Thus, a nuclear factor, henceforward referred to as GHF3, footprints a site within the rGH promoter (Fig. 1B) and is present in cells not expressing growth hormone, as well as cells in which rGH is expressed.
Two-dimensional Gel Shift Analysis of Multisubunit Transcription Factors

**Fig. 1.** A, protection of the rGH promoter between −239 and −219 against DNase I digestion after preincubation with 80 µg of GC (lanes 3, 7) or rat liver (LJV) (lanes 4, 8) nuclear extracts. −285/−198 fragments were labeled on either the antisense (lanes 1–4) or sense (lanes 5–8) strands. 0, no extract preincubation. G+A, Maxam-Gilbert sequencing ladder. B, sequence of the GHF3-binding site in the rGH promoter.

**Fig. 2.** A, mutations affecting GHF3 binding decrease rGH promoter activity. RNAs collected from GC cells transfected with RSVCAT and mutant or wild-type rGHCAT constructs are probed with an anti-sense rGHCAT SP6 transcript. The observed 275 nucleotide length of the band is exactly as predicted for transcription commencing from the authentic rGH start site. Internal control RSVfl-galactosidase transcripts are represented by a 255 length band spanning the CAT sequences. See Schaufele et al. for a more complete description of the mapping technique. Lane 1, 4X174 HaeIII size markers 271 and 281. B, the positions and sequences of the rGH promoter mutations analyzed are listed below the wild-type sequence, and their abilities to be bound (+) or not (0) by GHF3 or GHF4 factors are summarized.

-226, and −227,−219 mutations, which disrupted GHF3 factor binding, significantly decreased transcription from the rGH promoter (Fig. 2A, rGH, compare lanes 2 and 3 to the wild-type promoter, lane 1). Similar results were obtained by measuring CAT enzyme activity expressed from the linked rGH promoter (normalized to an RSV/β-galactosidase transfection control, data not shown). Quantitative densitometry of the RNase protection experiments revealed that correctly initiated transcripts were produced from the −230,−226 and −227,−219 promoters only 30% as efficiently as from the wild-type promoter. Thus, mutations disrupting the binding of a factor to the GHF3-binding site strongly correlated with reduced transcription from the rGH promoter.

**Multiple Factor Complexes Are Formed with the GHF3-binding Site**—Fragments spanning the GHF3-binding site were also analyzed for specific protein-DNA interactions by the gel mobility shift assay (Strauss and Varshavsky, 1984). A series of protein-DNA complexes were observed after the radiolabeled −237/−148 fragment was incubated with 4 µg of poly(dI-dC) and 8 µg of nuclear extract from the rat anterior pituitary GC cell line and separated on native polyacrylamide gels (Fig. 3, lane 2). Some of these complexes (labeled GHF3A–F) were more clearly resolved by other methods (see Fig. 8E). A similar gel shift pattern was also observed with extracts derived from rat liver, 235-1, Chinese hamster ovary, and HeLa cells (not shown). A 1000-fold molar excess of an unlabeled oligonucleotide containing the GHF3-binding site (Fig. 3, lane 3) competed effectively for almost all of these complexes (hereafter referred to as the GHF3 complexes) whereas oligonucleotides representing either the pGHF1- or GHF2 factor-binding sites within the rGH promoter (see Schaufele et al.) did not compete (lanes 4 and 5). All of the different GHF3 complexes copurified during DNA affinity chromatography with the GHF3 binding activity (compare with Fig 8E).

A single protein-DNA complex, GHF4, whose binding to the −237/−148 fragment was not competed by the GHF3-binding site oligonucleotide (lane 3) was affected by mutations within the −218 to −198 region of the rGH promoter (Fig. 2). These mutations had little or no effect on rGH promoter activity (Fig. 2A) under the cell culture and DNA transfection conditions used in these experiments.

Each mutation which disrupted GHF3 footprinting activity also disrupted the binding of all the GHF3 complexes detected by gel mobility shift (data not shown). Thus, the binding of each of these different complexes seemed to require similar specific sequences within this small segment of rGH promoter DNA, so we undertook to identify more precisely the binding location of each of these complexes by DNase I footprinting. Following incubation of the end-labeled −285/−198 fragment with GC cell extracts, the resulting complexes were mildly

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**Fig. 3.** Multiple gel shifted complexes formed with the −237/−148 fragment (lane 2) are competed with a 1000-fold molar excess of oligonucleotides (comp. oligo.) containing the GHF3 (lane 3) but not the pGHF1 or GHF2-binding sites (lanes 4 and 5). Lane 1, no GC cell nuclear extract added. Both GHF3 oligonucleotides shown in Fig. 7A compete similarly. Sequences of the pGHF1 and GHF2 oligonucleotides are found in Schaufele et al.
Two-dimensional Gel Shift Analysis of Multisubunit Transcription Factors

digested with DNase I before being separated by the gel mobility shift assay. Isolating three of the most predominant GHF3 complexes (GHF3-A, -B, -E, Fig. 3), extracting and rerunning the isolated DNAs on a sequencing gel (Fig. 4) demonstrated that the same nucleotides were protected against DNase I digestion in all three of the GHF3 complexes analyzed. This implies that a number of GHF3-binding species, each with a characteristic electrophoretic mobility, can bind to an identical rGH promoter site. In conclusion, maximal rGH promoter activity correlates with several nucleoprotein complexes each of which binds to the -239 to -219 region.

A ~43-kDa DNA-binding Protein Is Common to at Least Three of the GHF3 Complexes—Thus, at least three of the most prominent GHF3 complexes seem to recognize an identical sequence element (Fig. 4) but migrate differently on native polyacrylamide gels. The heterogeneous nature of the factors binding to this site could be a consequence of either (i) a family of distinct GHF3 factors with identical binding sites, (ii) differential modifications of a single factor, (iii) oligomerization of a single protein, (iv) the interaction of a single GHF3-binding factor with a number of different factors to form a series of heteromultimeric complexes, or (v) some combination of the above.

As a first step in the characterization of the GHF3 complexes, Southwestern blots (Singh et al., 1989) were performed to determine the size(s) of the protein(s) interacting with the GHF3-binding site. 64 µg of GC cell extract was fractionated by SDS-PAGE and the proteins were renatured after transfer to nitrocellulose. A ~43-kDa protein was strongly labeled after the resulting blot was probed with a radiolabeled oligonucleotide containing the GHF3-binding sequence (Fig. 5, GHF3). This protein was not detected when a radiolabeled oligonucleotide containing a binding site for the Spl transcription factor was used as a probe (Fig. 5, GHF2).

While the presence of a single, strongly labeled band on the Southwestern blot argues that a single protein may be interacting with the GHF3-binding site, other proteins were also weakly labeled. In order to further clarify whether a single or whether multiple DNA-binding proteins were responsible for the many different GHF3 complexes observed on gel mobility shift analysis, protein/DNA cross-linking experiments were performed. Three different GHF3 complexes formed with a radiolabeled 25-bp oligonucleotide corresponding to the DNase I-protected GHF3 site were isolated from gel shifts and exposed to ultraviolet light. Subsequent SDS-PAGE analysis of the proteins cross-linked to the GHF3 oligonucleotide by this procedure revealed a labeled protein common to all of the excised GHF3 complexes (Fig. 6). The size of the common cross-linked species was observed to be approximately 46 kDa (with cross-linked DNA) which agrees favorably with the predominant 43-kDa species seen on Southwestern blots (Fig. 5). Other proteins from the slower migrating complexes were also observed to be cross-linked to the GHF3 oligonucleotide, suggesting that some of the additional subunits present in the higher order complexes may also be contacting DNA. Therefore, at least some of the different GHF3 complexes seemed to contain in common a 43-kDa DNA-binding subunit (Fig. 6) which presumably accounts for the fact that some of the GHF3 complexes bind to the same DNA sequences (Fig. 4).

**Multimeric GHF3 Complexes Are Formed with Affinity Pu-**

![Fig. 4. Identical nucleotides are protected against DNase I digestion in the GHF3-A, -B, and -E complexes.](image)

![Fig. 5. Southwestern blot on GC extracts transferred to nitrocellulose using the kinased and ligated GHF3 oligonucleotide 2 (see Fig. 7A) as a probe.](image)

![Fig. 6. UV cross-linking of the 25-bp GHF3 oligonucleotide 1 (see sequence in Fig. 7A) to the GHF3-binding proteins reveals a protein common to each of the isolated GHF3 complexes.](image)
rified, Transcriptionally Active, GHF3—In order to ensure that the multimeric GHF3 complexes were specific and were not simply some artifact of the electrophoresis of crude extracts, the GHF3-binding activity was purified greater than 600-fold by DNA affinity chromatography (Kadonaga and Tjian, 1986; see "Materials and Methods"). Gel retardation analysis of the affinity enriched material still yielded multiple GHF3 complexes (see Fig. 8E, first dimension) suggesting that the interactions responsible for each of the GHF3 complexes can occur in solution and are stable.

To determine whether the enriched preparation contained transcriptionally active GHF3-binding factor, in vitro transcription studies were performed. Transcripts expressed from HeLa cell extracts which were depleted of GHF3-binding activity by DEAE-Sephacel chromatography (see "Materials and Methods") were analyzed by RNase protection assays (Fig. 7). Preincubation of template with partially purified GHF3-binding factor selectively stimulated transcription from those templates containing multiple copies of the GHF3-binding site inserted upstream of the rGH TATA box but not transcription from the same template devoid of GHF3-binding sites (−33rGH) nor from the coincubated RSV CAT template (Fig. 7B, compare the templates described in Fig. 7A with, +, or without, 0, preincubation with the GHF3-binding factor). Thus, the multimeric GHF3-binding factor is capable of stimulating the transcription of an RNA polymerase II promoter.

Diagonal Gel Mobility Shift Assays Reveal Relationships Among the Subunits of the Different GHF3 Complexes—In order to confirm the presence of a DNA-binding factor common to each of the GHF3 complexes, and to understand how individual subunits within each of these multimeric complexes may be related, a new technique, the diagonal gel shift, was devised (see Fig. 8 and "Materials and Methods"): a single lane from a gel mobility shift experiment performed with partially purified GHF3-binding protein was excised, layered horizontally overtop of a second, identical gel, and electrophoresed perpendicular to the original direction.

Assume for the moment that three different complexes were detected by gel mobility shift assays (shown schematically in Fig. 8A). If each of these complexes represented a unique monomeric protein then electrophoresis in the first dimension would displace each complex relative to one another such that electrophoresis in the second dimension would result in signals lying in a straight line diagonal to the two perpendicular directions of electrophoresis (see Fig. 8B). On the other hand, if each of the three complexes is represented by a multisubunit complex binding to the GHF3-binding site through a common DNA-binding subunit and if the protein-protein interactions establishing these multisubunit complexes are more labile than the protein-DNA interactions under the chosen electrophoretic conditions, then dissociation of the multisubunit complexes during the second dimension will result in the detection of lower complexity DNA-binding complexes lying below the diagonal (Fig. 8C). Only the DNA-binding subunits are followed since detection is by autoradiography of labeled DNA.

A pattern characteristic of multimeric GHF3 complexes was, in fact, observed (Fig. 8E). One-dimensional gel shifts to the left and above the diagonal gel serve to orient the location of the various signals with familiar landmarks, and a schematic representation of the locations of the putative complexes is also shown for clarity of explanation (Fig. 8F). Some of those signals falling off of the diagonal have an identical mobility in the second dimension (arrow, Fig. 8E) despite very different mobilities in the first dimension. This pattern suggests that a DNA-binding subunit is common to at least the GHF3-A, -B, -C, -D, and -E complexes.

Two GHF3 complexes, -C and -D, with slightly different electrophoretic mobilities in a unidimensional gel shift are clearly distinguished by the diagonal gel shift (Fig. 8E). A signal at the height of the GHF3-B complex lies directly below the position on the diagonal where the GHF3-D complex migrates. This strongly suggests that the GHF3-D complex consists of at least three subunits: the GHF3-A DNA-binding subunit, another subunit common to the GHF3-B complex and a further subunit (see Fig. 8D).

On the other hand, neither the GHF3-C nor the GHF3-E complexes dissociate into complexes containing the subunits common to the GHF3-B complex (Fig. 8E) suggesting that the GHF3-C and GHF3-E complexes likely do not represent higher order structures building further on to the GHF3-B or -D complexes. Rather the simplest interpretation of the GHF3-C and -E patterns is that they arise from an interaction of the GHF3-A DNA-binding subunit with a complement of subunits distinct from those in the GHF3-B complex. Furthermore, two signals (± and +, Fig. 8E) are observed to lie solely on the diagonal (+ is obscured in the longer exposure of the diagonal shown here and is poorly seen in the unidi-
DISCUSSION

The GHF3-binding Factor Is a Positive Regulator of rGH Transcription—Expression of the growth hormone gene is restricted to a defined subpopulation of cells within the anterior pituitary gland (Ivarie et al., 1983; Frawley et al., 1985). The pituitary-specific nature of growth hormone gene expression appears to be conferred in part by the binding of a positively regulating, pituitary-specific transcription factor (West et al., 1987; Catanzaro et al., 1987; Ye and Samuels, 1987; Lefèvre et al., 1987), Pit-1 (Ingraham et al., 1988), also called GHF1 (Bodner et al., 1988). There are two Pit-1-binding sites within the rat (West et al., 1987; Catanzaro et al., 1987) or human (Lefèvre et al., 1987; Bodner and Karin, 1987) growth hormone promoters. Because of the presence of Pit-1 mRNA and protein (Mangalam et al., 1988; Crenshaw et al., 1989) in cells in which the growth hormone promoter is inactive (Nelson et al., 1986; Behringer et al., 1988) other factors are probably important for the cell type-specific transcription of the growth hormone gene promoter. One candidate for this is the GHF7 factor found more specifically in GH expressing cell types.

An Sp1-binding site which overlaps the more distal Pit-1-binding site (Lemaigre et al., 1989) further contributes to the expression of the rat growth hormone gene promoter. We characterize here another transcription factor binding to sequences between −239 and −219 on the rGH promoter (Fig. 1), which positively regulates the growth hormone promoter since mutating its binding site decreases promoter strength (Figs. 2 and 7).

Fusion of rGH-producing cells with fibroblasts results in a rapid extinction of rGH gene expression (Lufkin and Bankert, 1987). Loss of rGH gene activity correlates with the disappearance of both a Pit-1 (McCormick et al., 1988; Triputti et al., 1988) and a GHF3-footprinting activity in nuclei from hybrid cells (Triputti et al., 1988) which led these authors to suggest that GHF3 binding may itself be tissue-specific and responsible for the cell-type-specific expression of the rGH gene (Triputti et al., 1988). Our data demonstrate that GHF3-binding activity is present in a variety of cell types which do not express rat growth hormone (Fig. 1) and that some rGH expression can occur in the absence of GHF3-binding activity (Fig. 2A). We therefore believe that the absence of GHF3-binding activity is not directly responsible for the extinction of rGH expression in those hybrid cells. GHF3 is rather more likely to be a general transcription factor aiding the expression of genes in several cell types.

In an effort to determine whether GHF3 shares identity with other previously characterized transcription factors, we have compared the GHF3-binding site with those compiled by Wingender (1988). A total of nine binding sites shared limited homology with the footprint region of GHF3 but these homologous regions extended only to five out of six contiguous nucleotides and corresponded to different portions of the GHF3-binding site (data not shown). Interestingly, of the nine partially homologous binding sites detected, three have been reported to bind factors (NF-κB, fos/jun, OTF-1) which form heteromultimers (Peterson and Calame, 1989; Chiu et al., 1988; Gerster and Roeder, 1988) but other biochemical and binding characteristics (Bohmann et al., 1987; Peterson and Calame, 1989; Strum et al., 1988) strongly suggests that none of these are GHF3. Thus, because of limited binding site homology, it is currently not possible to assign GHF3 to any of the known transcription factors.

* F. Schaufele, and T. L. Reudelhuber, unpublished results.
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A factor present in extracts from a number of different cell types was also previously decribed to bind to a fragment of the rGH promoter between -146 and -236 (Ye and Samuels, 1987). This factor was speculated to be involved in the thyroid hormone-mediated activation of the growth hormone gene (Flug et al., 1987). We note that the rGH promoters containing mutations which disrupt GHF3 binding, although reduced in promoter activity (Fig. 2), could still be potentiated by the addition of thyroid hormone. In addition, we detected no difference in the pattern or quantity of GHF3 nucleoprotein complexes formed when extracts are prepared from cells grown either in the presence or absence of thyroid hormone. Thus, the GHF3-binding site does not appear to be indispensable for the T3 induction of rGH, a result confirmed by others (Ye et al., 1988).

Diagonal Gel Mobility Shift Assays—Many different protein/DNA complexes were observed by gel mobility shift assays to be formed with the GHF3-binding site. These complexes were all competed by the same oligonucleotides, were affected by the same mutations, and footprinted the same sequences over the GHF3-binding site (Figs. 3 and 4 and data not shown). In order to further investigate the composition of these complexes, a rapid and simple technique for the elucidation of multimeric complexes containing a common DNA-binding subunit was developed. The procedure involves labeling one of the subunits with a subunit-specific ligand (in this case radiolabeled DNA) and observing the pattern in which other subunits dissociate from the DNA-binding subunit after electrophoresis in a second dimension; signals lying below points on a diagonal formed by electrophoresis in the second dimension represent subsets of the complexes on the diagonal which have lost one or more of the subunits. This dissociation is possibly aided by the low ionic strength of the gel running buffer and/or by the electric field itself since these complexes seemed to be more stable during their isolation by DNA affinity chromatography.

This diagonal gel shift technique was utilized to investigate the relationship between the seven different complexes formed with the GHF3-binding site within the rat growth hormone gene promoter which were observed by unidimensional gel mobility shift experiments. Five of the complexes had in common a DNA-binding subunit and two of these complexes even contained a second subunit in common (Fig. 8, E and P). This conclusion was confirmed by DNA-protein cross-linking experiments (Fig. 6) which demonstrated that a ~40 kDa (Fig. 5) DNA binding protein was common to at least three of the complexes. The present data suggests that a single DNA-binding protein could interact interchangeably with more than one other protein. Factors which bind to promoters through their association with DNA-binding proteins may have important consequences for transcriptional stimulation (see, for example, Schaffner 1989). The demonstrated interaction of a number of different proteins with the DNA-binding subunit in each of the GHF3 complexes could increase the plasticity of rGH promoter activities if each of the complexes to have different transcriptional effects. We believe that such a promoter construction would be relevant for fine tuning the level of transcription of a gene; the transcriptional effect of a physiological stimulus acting specifically on the formation of one of the complexes would be buffered by the activities of the other unaltered complexes. Further purification and characterization of each of the GHF3 complexes should allow us to test this hypothesis by assessing the relative effects of each on rGH promoter activity in our in vitro transcription system (Fig. 7).

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