The EMBO Journal vol.11 no.6 pp.2261 - 2269, 1992

Differential splicing of the *GHF1* primary transcript gives rise to two functionally distinct homeodomain proteins

Lars E.Theill[1], Kazue Hattori[1], Domenico Lazzaro[2], José-Luis Castrillo[1] and Michael Karin[1]

Department of Pharmacology[1], Center for Molecular Genetics, School of Medicine, University of California, San Diego, La Jolla, CA 92093-0636, USA and [2]EMBL Differentiation Programme, Postfach 10.2209, Meyerhofstrasse 1, D-6900 Heidelberg, Germany

Communicated by E.Wagner

The POU domain protein GHF-1 has a critical role in generation, proliferation and phenotypic expression of three pituitary cell types. GHF-1 functions in part by binding to and transactivating the promoters of both the growth hormone (*GH*) and prolactin (*PRL*) genes and that of the *GHF1* gene itself. We describe a naturally occurring isoform of GHF-1, GHF-2, in which an additional 26 amino acids are inserted into the activation domain of the protein as a result of alternative splicing. GHF-2 retains the DNA binding activity of GHF-1 and can activate the *GH* promoter but has lost the ability to activate the *PRL* and *GHF1* promoters. These results suggest that GHF-2 may function in differential target gene activation during differentiation of the somatotrophic lineage. Both GHF-1 and GHF-2 transcripts are specifically expressed in the anterior pituitary. Analysis of the genomic *GHF1* gene shows that most of the distinct functional domains of GHF-1 (and GHF-2) are encoded by separate exons. Gene segment duplication and exon shuffling may have contributed to the evolution of this cell type-specific transcriptional regulatory gene.

Key words: differential splicing/GHF1/growth hormone/homeodomain/transcription factor

Introduction

GHF-1 (Pit-1) is a pituitary specific homeodomain protein that belongs to the POU subclass (Bodner *et al.*, 1988; Ingraham *et al.*, 1988; Karin *et al.*, 1990). This family of transcriptional regulators shares a conserved DNA binding motif, the POU domain, composed of a 60 amino acid homeodomain (POU_{HD}) and a second region of ~75 amino acids located N-terminally to the homeodomain, the POU-specific domain (Herr *et al.*, 1988). POU domain proteins exert critical developmental and transcriptional functions (Finney *et al.*, 1988; Johnson and Hirsch, 1990; Finney and Ruvkin, 1990; Li *et al.*, 1990; Schöler *et al.*, 1990a,b; Verrijzer *et al.*, 1990; Castrillo *et al.*, 1991; Rosner *et al.*, 1991).

During mouse development GHF-1 protein is first detected on embryonic day 15, a time which coincides with activation of the growth hormone (*GH*) gene and expansion of the somatotrophic lineage (Dollé *et al.*, 1990; Simmons *et al.*, 1990). Transcription of the *GHF1* gene, on the other hand, is initiated 2 days earlier (Dollé *et al.*, 1990). Expression of both *GHF1* transcripts and protein is restricted to three cell types of the anterior pituitary, thought to be derived from a common progenitor (Dollé *et al.*, 1990; Simmons *et al.*, 1990). GHF-1 protein plays a critical role in expression and differentiation of the somatotrophic lineage (Li *et al.*, 1990; Castrillo *et al.*, 1991). Mutations in the *GHF1* gene cause pituitary dwarfism in the mouse. Such mice are deficient in both GH and PRL expression and exhibit anterior pituitary hypoplasia (Li *et al.*, 1990; Castrillo *et al.*, 1991).

Besides specific binding and transactivation of the *GH* and *PRL* genes (Bodner and Karin, 1987; Bodner *et al.*, 1988; Nelson *et al.*, 1988; Ingraham *et al.*, 1988; Theill *et al.*, 1989), GHF-1 positively autoregulates its own expression by binding to the promoter of the *GHF1* gene (McCormick *et al.*, 1990). With multiple functions in anterior pituitary cell proliferation, differentiation and polypeptide hormone gene activation, there may exist additional GHF-1 target genes involved in mediating these functions.

Differential activation of the *GH* and *PRL* genes occurs in GHF-1 expressing cells (Bodner *et al.*, 1988; Dollé *et al.*, 1990; Simmons *et al.*, 1990; Castrillo *et al.*, 1991). During development, PRL expression is first detected post-natally, 14 days after the onset of GH expression (Slabaugh *et al.*, 1982). Although most of the prolactin-expressing cells appear to be derived from GH-producing precursors (Chatelain, 1979; Behringer *et al.*, 1988; Borrelli *et al.*, 1989), only a small number of cells simultaneously express both GH and PRL. Additional activating and restricting mechanisms must therefore be required for differential and selective expression of these and other GHF-1 target genes during pituitary development and differentiation. One such mechanism may involve cooperation between GHF-1 and other transcriptional activators that bind to the promoters or enhancers of various GHF-1 target genes (Crenshaw *et al.*, 1989; McCormick *et al.*, 1990; L.E.Theill, unpublished results). Generation of variant transcription factors by differential splicing of a simple primary transcript could provide an additional mechanism for differential gene regulation. For example, a pituitary-specific isoform of the thyroid hormone receptor (r-*erb*Aβ-2) present in somatotrophic cells was identified (Hodin *et al.*, 1989). We now present the isolation and characterization of a naturally occurring variant of GHF-1 and show that this protein, named GHF-2, can activate only a subset of the known GHF-1 target genes. To trace the origin of GHF-2, we isolated and determined the structure of the rat genomic *GHF1* gene. GHF-2 is generated by differential splicing of the *GHF1* primary transcript. Like GHF-1, GHF-2 is also exclusively expressed in the anterior pituitary.

Results

Isolation of rat GHF-2 cDNA clones

The first evidence for an isoform of GHF-1 came from screening of a rat pituitary tumor cell line cDNA library in search of GHF-1 cDNA clones (Bodner *et al.*, 1988). One

out of six cDNA clones that hybridized to the GHF-1 oligonucleotide probes, carried an insertion of 78 bp after nucleotide 142 relative to the initiator ATG codon of the reported GHF-1 cDNA sequence (Figure 1). To investigate further the natural occurrence and structure of this GHF-1 isoform we employed PCR techniques. Oligodeoxynucleotides complementary to the 5′- and 3′-ends of the rat GHF-1 coding sequence were synthesized and used to amplify specifically GHF-1 coding sequences from rat pituitary tumor cell line (GC) cDNAs primed with random oligonucleotides. A major PCR product of 0.9 kbp as well as a minor 0.95 kbp product were visible after separation by agarose gel electrophoresis (unpublished data). Isolation and sequence analysis confirmed that the 0.9 kbp fragment was identical to the rat GHF-1 cDNA previously described (Bodner *et al.*, 1988). The rat 0.95 kbp cDNA fragment was identical to the variant cDNA clones described above. The protein encoded by the variant sequence was named GHF-2 and is identical to GHF-1 except for an in-frame insertion of 26 amino acids following amino acid 47 of GHF-1. Interestingly, the insertion of GHF-2 is in the middle of the previously defined GHF-1 transactivation domain (STA domain), which is rich in hydroxylated amino acid residues (Theill *et al.*, 1989).

```
ATG AGT TGC CAA CCT TTC ACC TCG GCT GAT ACC TTT ATA
 M   S   C   Q   P   F   T   S   A   D   T   F   I

CCT CTG AAT TCT GAC GCT TCT GCT GCC CTG CCT CTG AGA
 P   L   N   S   D   A   S   A   A   L   P   L   R

ATG CAC CAC AGT GCC GCT GAG GGT CTC CCA GCC TCC AAC
 M   H   H   S   A   A   E   G   L   P   A   S   N

CAC GCC ACC AAC GTG ATG TCC ACA G gtactgacct ... ttcttaccag
 H   A   T   N   V   M   S   T

 TC CCG TCT ATT TTG TCT TTG ATC CAA ACA CCT AAA TGT
 V   P   S   I   L   S   L   I   Q   T   P   K   C

TTG CAC ACA TAT TTC TCG ATG ACA ACG ATG GGA AAT ACA
 L   H   T   Y   F   S   M   T   T   M   G   N   T

G    CG ACA GGA CTT CAT TAT TCT GTG CCT TCC TGT CAT
      A   T   G   L   H   Y   S   V   P   S   C   H

TAT GGA AAC CAG CCA TCC ACC TAC GGA GTG ATG GCA G
 Y   G   N   Q   P   S   T   Y   G   V   M   A

gtaagaaaca  ttctgcacag GC ACT TTA ACC CCT TGT CTT TAC AAG
                        G   T   L   T   P   C   L   Y   K

TTT CCA GAC CAC ACC CTG AGT CAT GGG TTT CCT CCC CTG
 F   P   D   H   T   L   S   H   G   F   P   P   L

CAC CAA CCT CTG CTG GCG GAG GAC CCA ACA GCC TCT GAA
 H   Q   P   L   L   A   E   D   P   T   A   S   E

TTT AAG CAG GAA CTC AGG CGG AAA AGT AAA TTG GTG GAA
 F   K   Q   E   L   R   R   K   S   K   L   V   E

GAG CCA ATA GAC ATG GAC TCC CCG GAA ATC CGA GAA CTG
 E   P   I   D   M   D   S   P   E   I   R   E   L

GAG CAG TTT GCC AAC GAA TTT AAA GTG AGA AGA ATT AAG
 E   Q   F   A   N   E   F   K   V   R   R   I   K

TTA G gtacgccttt      cctctcacag GA TAC ACC CAG ACA AAC GTG
 L                           G   Y   T   Q   T   N   V

GGC GAA GCT CTG GCC GCT GTC CAC GGC TCC GAA TTC AGT
 G   E   A   L   A   A   V   H   G   S   E   F   S

CAA ACA ACC ATC TGC CGA TTT GAA AAC TTG CAG CTC AGT
 Q   T   T   I   C   R   F   E   N   L   Q   L   S

TTC AAA AAT GCT TGC AAA CTG AAA GCA ATT TTA TCC AAG
 F   K   N   A   C   K   L   K   A   I   L   S   K

TGG CTG GAG GAA GCT GAG CAG GTC GGA G gtactgaagc cacatttcag
 W   L   E   E   A   E   Q   V   G

 CT TTG TAC AAT GAA AAA GTG GGA GCA AAC GAA AGG AAG
 A   L   Y   N   E   K   V   G   A   N   E   R   K

AGG AAA CGG AGG ACA ACT ATC AG gtatttctc  cctcccccag T
 R   K   R   R   T   T   I   S

ATC GCC GCT AAG GAT GCT TTG GAG AGA CAC TTT GGA GAG
 I   A   A   K   D   A   L   E   R   H   F   G   E

CAC AGC AAA CCT TCT TCG CAG GAG ATC ATG CGG ATG GCT
 H   S   K   P   S   S   Q   E   I   M   R   M   A

GAA GAA TTG AAT CTC GAG AAA GAA GTA GTA AGA GTG TGG
 E   E   L   N   L   E   K   E   V   V   R   V   W

TTT TGC AAC CGA AGG CAG AGA GAA AAA CGG GTG AAA ACG
 F   C   N   R   R   Q   R   E   K   R   V   K   T

AGT CTC AAT CAA AGT CTG TTC TCT ATT TCA AAG GAG CAT
 S   L   N   Q   S   L   F   S   I   S   K   E   H

CTT GAG TGC AGA TAA
 L   E   C   R   *
```

Fig. 1. Sequence of rat GHF-2 cDNA combined with the position of the intron–exon boundaries of the rat genomic *GHF1* gene. The first and last 10 nucleotides of each intron are indicated in lower case letters. The sequence unique to rat GHF-2 cDNA is boxed. The exact same sequence was present at the 3′-end of intron a as determined by sequencing the genomic *GHF1* gene. The predicted amino acid sequence is shown in the single letter code below the nucleotide sequence.

GHF-1 and GHF-2 are differentially spliced products of the same gene

The presence of the in-frame 26 amino acid insertion in GHF-2 suggested that it could be derived from an alternatively spliced form of the *GHF1* primary transcript. To explore this possibility and to determine its genomic

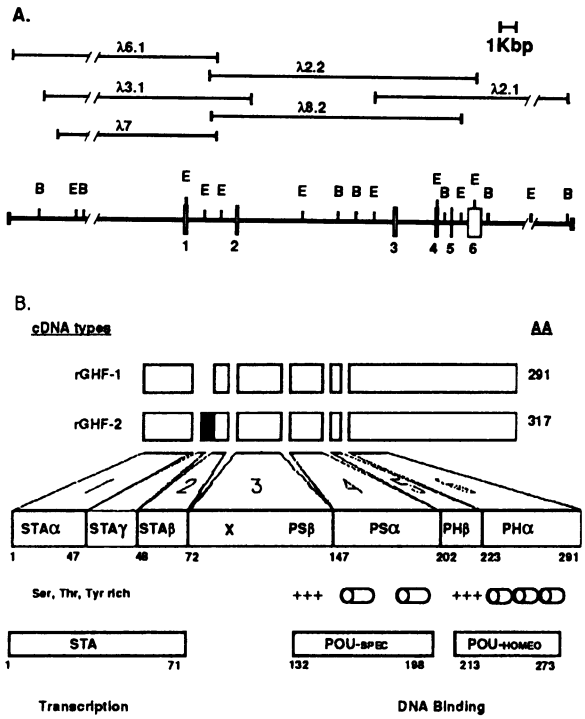

Fig. 2. Structure of the *GHF1* gene. (**A**) The organization of λ clones containing *GHF1* gene fragments is indicated. The restriction map of a 21 kbp region of the rat genome encompassing the *GHF1* gene is shown (E, *Eco*RI; B, *Bam*HI). The exons are denoted by the numbered open boxes. (**B**) Comparison of the *GHF1* exon organization to the structure of GHF-1. Exons present in GHF-1 cDNA are denoted by open boxes. The insert in the GHF-2 cDNA is denoted by a solid box. The amino acid positions corresponding to the exon borders are indicated on the schematic GHF-1 protein sequence. (+++) indicate motifs rich in basic amino acids. The cylinders indicate potential α-helical regions. STA is an abbreviation for Serine, Threonine and Tyrosine-rich Activating domain. Numbers below the boxes indicate the amino acid positions.

organization, the rat *GHF1* gene was isolated and characterized. Six independent overlapping clones spanning an area of 43 kbp and containing the complete GHF-1 coding region were isolated from a rat genomic library. The restriction enzyme map of this area and the genomic structure of *rGHF1* are presented in Figure 2A. The nucleotide sequences of all exons and introns except for 6 kbp of intron b, 0.5 kbp of intron C and 0.5 kbp of intron d, a total of more than 12 kbp, were determined and are available through the EMBL Data Library. The sequence of the 5′-flanking region of the gene as well as the promoter structure and start site of transcription were reported previously (McCormick *et al.*, 1990). The *GHF1* gene consists of six exons and five introns. The six exons code for amino acids 1−47, 48−71, 72−146, 147−201, 202−222 and 223−291 of the rat GHF-1 sequence, respectively (Figures 1 and 2B). The length of introns a to e are 2.6, 9.8, 2.3, 1.0 and 0.8 kbp respectively. All of the splice donor and acceptor sites follow the GT−AG rule (Mount, 1982; Figure 1). These exon−intron boundaries are identical to the ones found in the mouse *GHF1* (*Pit1*) gene (Li *et al.*, 1990).

Sequencing of the genomic *GHF1* gene shows that the 78 bp insertion in rGHF-2 mRNA is generated by the use of an alternative splice acceptor site in intron a, 78 bp upstream of the one used in the case of rGHF-1 (Figures 1 and 2B). Interestingly the splice acceptor site used for rGHF-2 fits the acceptor site consensus sequence better than does the site used to generate rGHF-1 (Ohshima and Gotoh, 1987; Figure 3).

Transactivation properties of GHF-2 are distinct from GHF-1

We have previously shown that deletions in the STA domain abolish GHF-1's ability to transactivate the *GH* promoter (Theill *et al.*, 1989). Identification of an isoform of GHF-1 with an interrupted STA domain prompted us to examine the ability of GHF-2 to activate the target genes so far identified for GHF-1. In titration DNase I footprinting experiments 10 ng of either recombinant GHF-1 or GHF-2 proteins gave full and identical protection of the two GHF-1 binding sites present in the *GH* promoter (Figure 4). To examine the relative binding affinity of GHF-2 to known GHF-1 binding sites competition experiments were performed using radiolabelled oligonucleotide corresponding to the high affinity site of the *rGH* gene and unlabelled oligonucleotides corresponding to the same site (rGH-P), a mutated version of the same site (mutrGH-P) and the high affinity binding sites of the *GHF1* (rGHF1-P) and *PRL* (rPRL-1P) genes. The competition curves observed for GHF-1 and GHF-2 were very similar such that the rGH-P, rGHF1-P and rPRL-1P oligonucleotides but not the mutrGH-P oligonucleotide competed readily for binding to the labelled rGH-P probe (Figure 5). These results indicate that the DNA binding affinity of both GHF-1 and GHF-2 for all three sites are relatively similar. GHF-1 and GHF-2 therefore appear to have identical DNA binding activities.

Exogenous GHF-1 and GHF-2 proteins of the predictable mobility were present in similar amounts in nuclei of RQ6 cells transfected with either RSVGHF1 or RSVGHF2

GHF1 intron a:	c g a t g g g a a a t a c a g CGACA
GHF2 intron a':	t a c t t t t c t t a c c a g TCCCG
consensus splice acceptor site:	y y y y y y y y y y y y a g RNNGY C

Fig. 3. Comparison of the splice acceptors used for generation of GHF-1 and GHF-2. *GHF1* gene sequences around the intron a and a′ 3′-splice junctions, used for splicing of the primary transcript to generate the GHF1 and GHF2 mRNAs, respectively, are shown. Nucleotides matching the vertebrate splice acceptor site consensus sequence (Ohshima *et al.*, 1987) are bold faced. Lower case lettering indicates intron sequences. Y, pyrimidines; R, purines; N, any nucleotide.

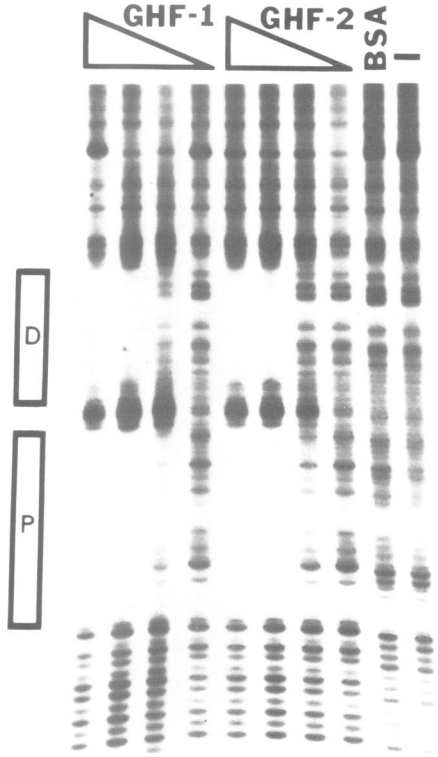

Fig. 4. DNase I footprinting by GHF-1 and GHF-2. An end-labelled *hGH* probe (+3 to −500, labelled at +3) was incubated in parallel with increasing amounts (1, 4, 10 and 40 ng) of recombinant GHF-1 and GHF-2 proteins and was subjected in DNase footprinting. The locations of the proximal (p) and distal (d) GHF-1 binding sites in the *hGH* promoter are indicated on the side panel.

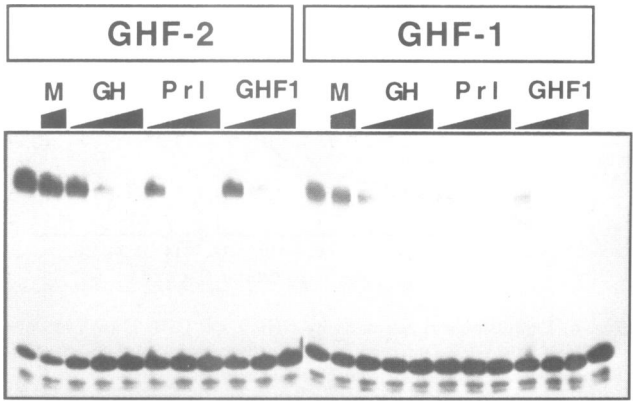

Fig. 5. Mobility shift assays of GHF-1 and GHF-2. 0.3 nM radiolabelled rGH-P site was incubated with recombinant GHF-1 (3 ng) or GHF-2 (6 ng) in the presence of increasing amounts (6, 30 or 120 nM) of unlabelled mutGH-P, (M); rGH-P, (GH); rGHF1-P, (GHF-1); or rPRL-1P, (PrL) sites. A representative set of experiments is shown. Both gels were run in parallel.

expression vectors (Figure 6A). In the Rat6 cell line GHF-1 and GHF-2 were equally effective in activating a cotransfected h*GH*-CAT reporter gene (Figure 6B). Both factors also activated a mutated *GH* promoter containing only one GHF-1 binding site, Δ121−126h*GH*-CAT, whereas they fail to activate a *GH* promoter from which the GHF-1 binding sites were deleted, Δ82−128h*GH*-CAT (Figure 7). Besides the *GH* promoter, GHF-1 is also an activator of the *PRL* and *GHF1* promoters (Ingraham *et al.*, 1990; McCormick *et al.*, 1990; Castrillo *et al.*, 1991; and Figure 7). GHF-2 on the other hand, while an effective activator of the *GH* promoter, failed to activate either the *PRL* or *GHF1* promoters (Figure 7). These findings indicate that GHF-1 and GHF-2 have different, yet overlapping, target gene specificities: GHF-2 activates only the *GH* promoter while GHF-1 activates the promoters of all three genes: *GH*, *PRL* and *GHF1*. The fact that deletion of one of the GHF-1 binding sites in the *GH* promoter did not abolish GHF-2 transactivation indicates that it is not the number of binding sites that determines whether GHF-2 can activate a given promoter or not. Unlike the *GH* promoter, the *GHF1* promoter region used in these experiments contains only a single high affinty *GHF1* binding site (McCormick *et al.*, 1990) to which both proteins bind with similar affinities (see Figure 5).

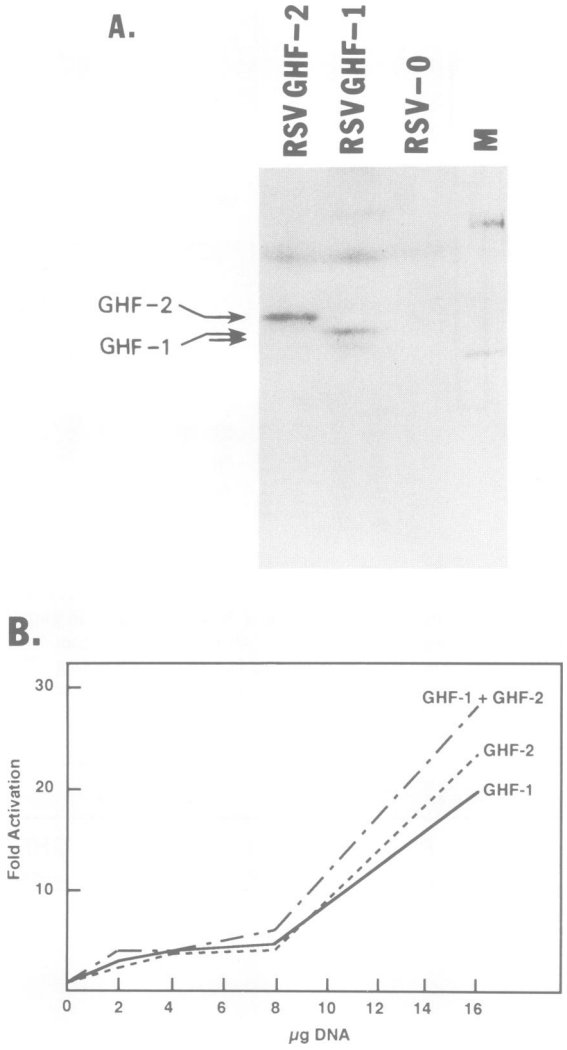

Fig. 6. Expression and activation by GHF-1 and GHF-2. (**a**) Detection of GHF-1 and GHF-2 proteins in transiently transfected RQ6 cells. RQ6 cells were transfected with 20 μg of either RSVGHF1, RSVGHF2 or the empty expression vector RSV-0 and analyzed by Western blotting using anti-GHF1 antibodies. (**B**) GHF-1 and GHF-2 activate the GH promoter. The indicated amounts of the GHF-1 and GHF-2 expression vectors or a one to one combination of the two (GHF-1 + GHF-2) were cotransfected into Rat6 cells with 5 μg of 289hGH−CAT reporter construct. The amount of RSV expression vector in each transfection was adjusted to 16 μg by the addition of RSV-0. Shown are the averages of two different experiments.

Expression of GHF-2

With the identification of differential target gene specificity for GHF-1 and GHF-2 it became important to examine the level of expression of GHF-2 mRNA and protein in pituitary cells and to test if there are differences in the pattern of GHF-1 and GHF-2 expression. Total RNA was isolated from rat pituitaries, embryos and cell lines and the relative level of GHF-2 versus GHF-1 transcripts was analyzed by RNase protection. The 0.6 kb long probe contains 517 nucleotide complementary to GHF-2 mRNA and spans the 78 nucleotide region not present in the GHF-1 mRNA. As a result 517 and 346 nucleotide fragments should be protected by the GHF-2 and GHF-1 transcripts, respectively (Figure 8A). Both transcripts were detected in the pituitaries of male and pregnant female rats as well as in the GC, GH_3 and 235-1 pituitary tumor cell lines (Figure 8B). In addition both transcripts were found in rat embryos from gestational days 17 and 19 (data not shown). The relative levels of the GHF-2 and GHF-1 transcripts were quantitated by an imaging system that directly quantitates radiation (AMBIS™). In all pituitaries and cell lines positive for GHF-1 the ratio of GHF-2 to GHF-1 transcripts was ~1−7 (see legend to Figure 8B).

GHF-2 expression during rat embryogenesis and in adult

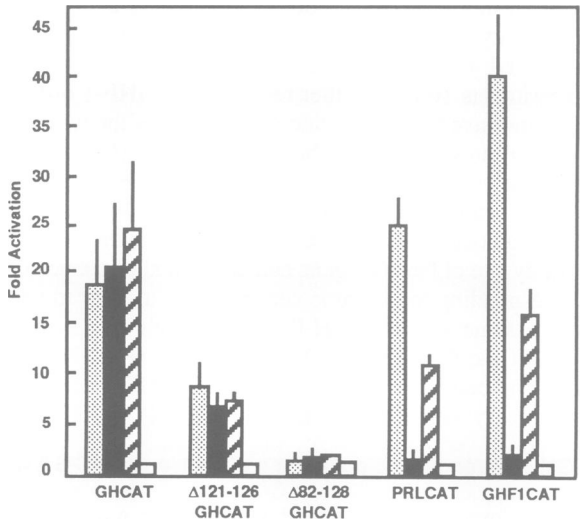

Fig. 7. GHF-1 and GHF-2 differ in their transactivation properties. 20 μg of GHF-1 (dotted column), GHF-2 (solid column), a mixture containing 10 μg of each (hatched column) or 20 μg of empty expression vector (empty column) were cotransfected into Rat6 cells with 5 μg of the following reporter constructs: −289hGH−CAT; Δ121−126hGH−CAT (distal GHF-1 binding site deleted); Δ82−128hGH−CAT (both GHF-1 binding sites deleted); −415 PRL−CAT and −200 GHF-1−CAT. The cells were analyzed for CAT activity 48 h after transfection. Fold activation was determined relative to the basal expression of each reporter plasmid, and represents the average of three separate experiments.

rat pituitaries was further analyzed by *in situ* hybridization with a riboprobe derived from the 78 nucleotide GHF-2 specific sequence. *In situ* analysis of serial sections of rat embryos from 12.5 and 13.5 days postcoitum (p.c.) revealed no specific signal. GHF-2 mRNA were first clearly detected by day 15.5 p.c., when a specific signal was observed throughout the anterior pituitary (Figure 9A). In isolated adult pituitaries GHF-2 transcripts were also restricted to the anterior lobe (Figure 9B). The pattern of expression of GHF-1 (Dollé *et al.*, 1990; Simmons *et al.*, 1990) and GHF-2 transcripts thus appears to be similar.

Expression of GHF-2 protein was first examined by immunoblotting GC cell nuclear extracts separated by SDS−PAGE. In addition to the 33 and 31 kDa bands corresponding to GHF-1 (Bodner *et al.*, 1988; Castrillo *et al.*, 1989), a faint band of ~35 kDa which is the predictable size of GHF-2, was observed (data not shown). Due to its low abundance, it was difficult to visualize the GHF-2 band, and therefore, we have used mobility shift assays to examine whether pituitary cell extracts contain a DNA binding activity with similar mobility to that of GHF-2. Complexes with similar mobilities to the ones generated by recombinant GHF-1 and GHF-2 were observed in extracts of pituitary cell lines (Figure 10A). Due to the small difference in the electrophoretic mobilities of GHF-1 and GHF-2, it was not possible to determine clearly the origin of the complexes generated by the pituitary cell extract. However, extended electrophoretic separation revealed that the top of the protein−DNA complex formed by the pituitary cell extract migrated more slowly than the GHF-1

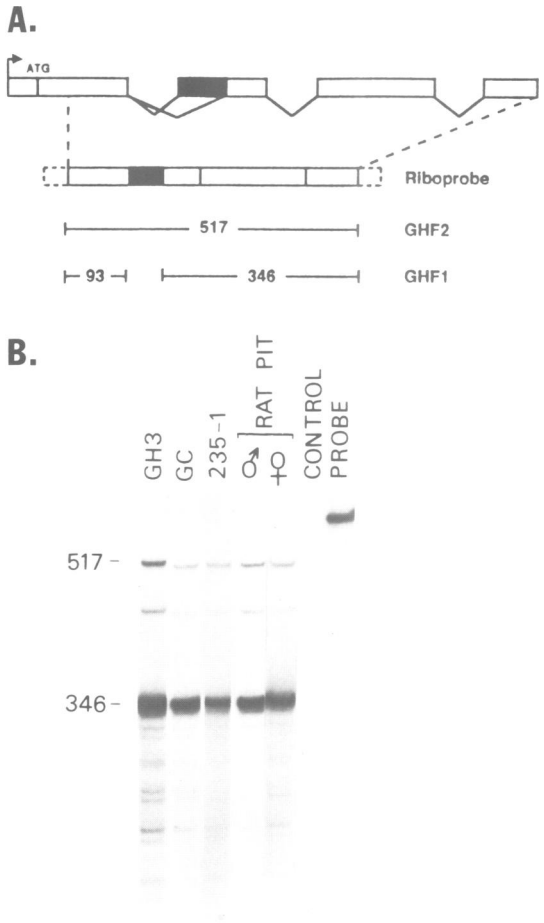

Fig. 8. Expression of GHF-1 and GHF-2 transcripts. (**A**) Schematic representation of the RNase protection experiment. Exons and introns from only the first half of the *GHF1* gene are shown. Exon sequences common to GHF-1 and GHF-2 (open boxes) or specific for GHF-2 (solid box) are indicated, as well as the expected lengths of the protected fragments. (**B**) Expression of GHF-2 relative to GHF-1 transcripts in rat pituitary tissue and pituitary tumor cell lines. RNase protection experiments were performed using total RNA and a radioactive antisense RNA probe described in panel A. The RNA sources are indicated above the lanes. Pituitaries were dissected from either young male or pregnant female rats. Various amounts of total RNA (5−30 μg) and autoradiography times were used to generate signals of similar intensity. The quantitated ratio, adjusted for fragment size, of the GHF-1 to GHF-2 signals was as follows: GH_3, 6.7; GC, 7.0; 235, 7.0; rat pituitary, 7.1.

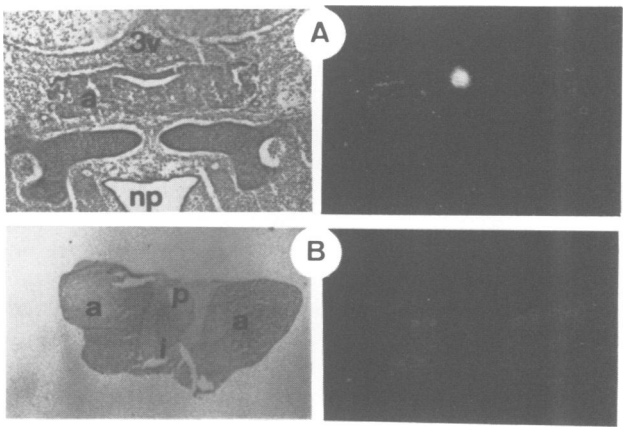

Fig. 9. GHF-2 transcript expression pattern in rat fetal and adult pituitary. Sections were hybridized to the GHF-2 riboprobe and photographed under bright-field (left) and dark-field (right). (**A**) Section in a frontal plane through the region containing the anterior pituitary of rat fetus day 15.5 p.c. The bright spot in the centre of the darkfield frame is an artefact and does not represent a specific hybridization signal. (**B**) Adult rat pituitary in a frontal orientation. GHF-2-specific signal is observed only in the anterior lobe. a, anterior lobe; i, intermediate lobe; p, posterior lobe; 3v, third ventricle; np, nasopharyngial cavity.

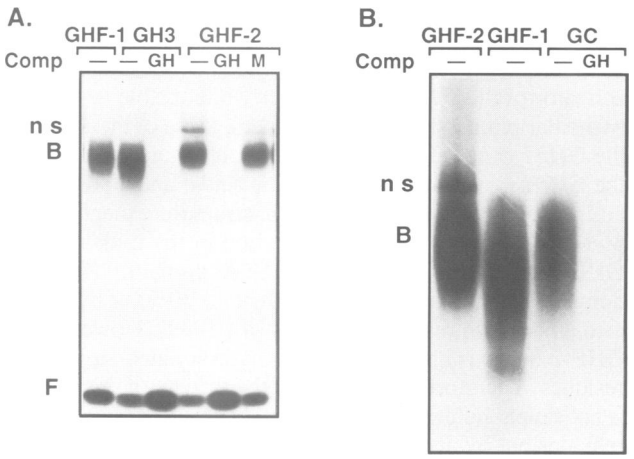

Fig. 10. Electrophoretic mobility shift analysis of GHF-1, GHF-2 and pituitary cell extracts. (**A**) 0.3 nM radiolabelled rGH-P site was incubated with GHF-1 (4 ng), GHF-2 (4 ng) or GH_3 whole cell extract (1.5 μg) in the absence (−) or presence of 80 nM rGH-P (GH) or mutGH (M) GHF-1 binding site. (**B**) As in A, but long gel run, and using GC whole cell extract 1 μg. n.s., non-specifically bound probe.

protein–DNA complex, at a position similar to that of the GHF-2 protein–DNA complex (Figure 10B). These results suggest that GHF-2 contributes to the total GHF-1 binding activity present in pituitary cell extracts.

Splicing pattern of a chimeric GHF-1/α-globin transcript

The splice acceptor site used to generate GHF-2 adheres more closely to the consensus splice acceptor site (Ohshima and Gotoh, 1987) than does the sequence used for generating GHF-1 (see Figure 3). It was possible, therefore, that the splicing pathway leading to formation of GHF-1 is dependent on an active mechanism conferred by a cell-type specific factor. To test this hypothesis, we analyzed the transcription and splicing of the entire genomic *GHF1* gene under control of the MT promoter after transfection into a heterologous cell line. Both 517 and 346 nucleotide protected fragments representing GHF-2 and GHF-1 transcripts, respectively (Figure 8A), were detected (Figure 11B, lane 4). However, due to the large size of this construct, the transfection efficiency was low resulting in high background. To circumvent this problem, we constructed and studied by RNase protection the expression and splicing of a smaller chimeric *rGHF1/α-globin* gene construct (MGαG) containing *GHF1* exon I, intron a and exon II. The GHF-2 riboprobe described above should generate a 243 and a 93 nucleotide protected fragments that reflect the use of the rGHF-2 and rGHF-1 splice acceptor sites, respectively (Figure 11A). Both transcripts were detected after transfection of MGαG into RQ6, HeLa and F9 cells (Figure 11B) and Rat6 cells (unpublished data). Quantitation indicated a splicing ratio between the GHF-2 and GHF-1 forms of the chimeric transcript of ~1:7 in all cell lines (see legend to Figure 11). In conclusion, formation of the GHF-1 and GHF-2 transcripts does not appear to depend on cell type-specific splicing factors.

Discussion

In the present study we have identified a naturally occurring isoform of the pituitary-specific transcription factor GHF-1 which arises from alternative splicing of the primary *GHF1* gene transcript. This isoform, GHF-2, is an efficient activator of the *GH* gene promoter while unable to activate two other pituitary-specific promoters, those of the *PRL* and the *GHF1* genes. The two transcription factors produced by the *GHF1* gene, GHF-1 (291 amino acids) and GHF-2 (317 amino acids) are identical in their structure except for the presence of a 26 amino acid insertion in the middle of the GHF-1 activation domain, the STA domain. The STA domain is signified by its high content (≥30%) of hydroxylated amino acid residues (Theill *et al.*, 1989). However, the GHF-2 insert is also quite rich in hydroxylated amino acid residues. Therefore it appears that the activity of this domain is not simply influenced by the overall content of hydroxylated residues but by another structural feature which remains to be determined.

The limited activation potential of GHF-2 is not determined by the number of GHF-1 binding sites within the target promoter. Deletion of one of the two GHF-1 binding sites of the *GH* promoter did not abolish its response to GHF-2. Both the *GHF1* promoter with a single high affinity GHF-1 binding site (McCormick *et al.*, 1990) and the *PRL* promoter containing four moderate affinity GHF-1

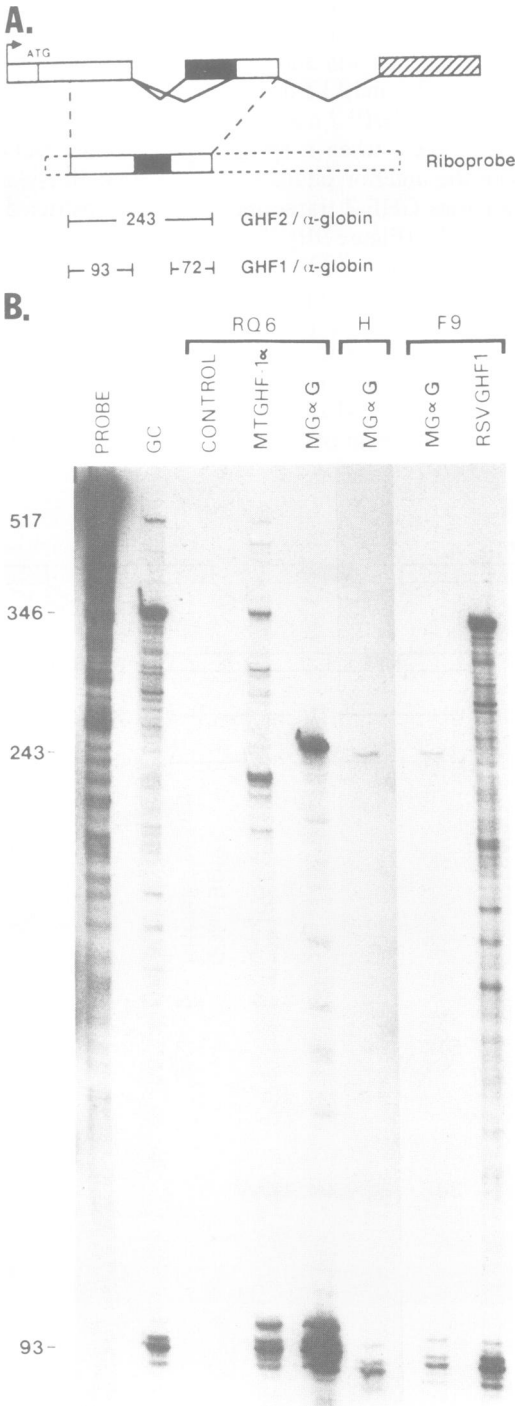

Fig. 11. Expression and splicing of a chimeric GHF1/α-globin transcript. (**A**) Schematic representation of the RNase protection analysis of cells transfected with MGαG. Exon sequences common to GHF-1 and GHF-2 (open boxes) or specific for GHF-2 (solid box) as well as α-globin exon 2 sequences (hatched box) are indicated, as well as the expected lengths of the protected fragments. For a schematic representation of the RNase mapping experiments with GC cells and cells transfected with MTGHF-1α or RSVGHF1 see Figure 8(A). (**B**) Expression of both GHF-1 and GHF-2 splice-forms in transiently transfected heterologous cell lines. RQ6, HeLa (H) and F9 cells were transfected with 20 μg MGαG plasmid DNA. RQ6 cells were also transfected with the MTGHF-1α expression vector. F9 cells transfected with the expression vector RSVGHF1 that only expresses GHF-1 transcripts are included as a GHF-2 negative control. RNase protection experiments were performed using total RNA and the riboprobe described in (A). The RNA sources are indicated above the lanes. The GHF-2 to GHF-1 splicing ratio was calculated for transcripts derived from pMGαG transfected cells: RQ6, 7.0; HeLa, 6.4; F9, 7.1.

binding sites (Nelson *et al.*, 1988) are not activated by GHF-2. This selective promoter activation by GHF-2 is not due to differential binding, because it binds to the *GH* and *GHF1* promoters as efficiently as GHF-1. One possibility is that GHF-2 is an effective activator only in collaboration with other sequence-specific transcription factors that bind to the *GH* promoter but not to the *GHF1* and *PRL* promoters. It is possible that the insert within the GHF-2 activation domain alters its conformation and thereby affects its ability to interact with target proteins that can be either sequence-specific activators or components of the basic transcriptional machinery, which are required for efficient activation of the *GHF1* and *PRL* promoters. GHF-1, on the other hand, can interact with accessory proteins that bind all three promoters.

Due to the different activation properties of GHF-2, the differential splicing of the *GHF1* primary transcript may be one of the mechanisms contributing to the selective activation of GHF-1 target genes during anterior pituitary development (Bodner *et al.*, 1988; Dollé *et al.*, 1990; Simmons *et al.*, 1990). The GH-producing somatotrophs and PRL-producing lactotrophs arise from the same progenitor (Chatelain, 1979) and they both contain GHF-1 immunoreactive material (Bodner *et al.*, 1988). GHF-1 is required for generation of both cell types (Li *et al.*, 1990; Castrillo *et al.*, 1991) and for activation of both the *GH* and *PRL* genes (Bodner and Karin, 1987; Nelson *et al.*, 1988; Ingraham *et al.*, 1988; Castrillo *et al.*, 1991). The initial appearance of GHF-1 protein shows temporal and spatial correlation with GH expression (Dollé *et al.*, 1990; Simmons *et al.*, 1990). On the other hand, no significant PRL expression can be detected until 10−12 days postnatally (Slabaugh *et al.*, 1982; Dollé *et al.*, 1990). The lack of GH expression in lactotrophs and the lack of PRL expression in somatotrophs, both of which contain GHF-1, has remained an enigma (Karin *et al.*, 1990). It is possible that changes in the levels of GHF-1 and GHF-2 production may contribute to the differential activation of the *GH* and *PRL* genes. At the present time, however, we have not been able to obtain direct evidence for differential expression of GHF-1 and GHF-2 during pituitary development and thorough examination of this point will require the preparation of GHF-2-specific antibodies for use in immunohistochemical analysis. Alternatively, such studies would require the development of techniques for separation of somatotrophs and lactotrophs which are currently not available. In the samples examined in the study we have observed a more-or-less constant ratio of 1:7 between GHF-2 and GHF-1 transcripts. However, most of the cell types that were examined for GHF-1 and GHF-2 expression were kept under standard experimental conditions and it is perfectly possible that the ratio of the two transcripts varies in response to yet-to-be determined experimental conditions or hormonal stimuli.

The importance of alternative splicing as a developmental regulatory mechanism was demonstrated in *Drosophila* where differential splicing underlies sex determination (see Baker, 1989 for a review). Alternative splicing was reported for two other members of the POU subfamily, the *Oct2* (Hatzopoulos *et al.*, 1990; Wirth *et al.*, 1991) and *Oct4* (Schöler *et al.*, 1990b) genes. The ratios of Oct-2 isoforms were found to be similar in different cell types (Wirth *et al.*, 1991). Differences were observed, however, in the pattern of expression of Oct-2a and Oct-2b (Hatzopoulos *et al.*, 1990). It remains to be seen whether the different Oct-2 proteins function similarly or differently from one another.

Alternative splicing affecting the coding region has also been described for other homeogenes, including *ultrabithorax* (O'Connor *et al.*, 1988) and *labial* (Mlodzik *et al.*, 1988). Differential splicing affecting the transactivation domain was reported for CREB (Yamamoto *et al.*, 1990; Berkowitz and Gilman, 1990), CTF/NF-1 (Mermod *et al.*, 1989), thyroid hormone receptor (Izumo and Mahdavi, 1988; Hodin *et al.*, 1989), retinoic acid receptors (Leroy *et al.*, 1991; Zelent *et al.*, 1991) and the E2 (Lambert *et al.*, 1987) and E1A (Lillie *et al.*, 1986) regulatory proteins of adenovirus. Differential cell-specific splicing patterns as well as differences in the abilities of the various isoforms to activate or repress transcription have been observed in some of these studies. However, in no case known to us, was it demonstrated that two different isoforms generated by alternative splicing are capable of differential target gene activation, as shown for GHF-1 and GHF-2.

The domain structure of GHF-1 correlates with the gene structure

In comparing the structure of the *GHF1* gene with that of the GHF-1 protein we find a relatively good correlation between the exon structure and the distribution of functional domains within the protein. The GHF-1 activation domain which resides between amino acids 1 and 71 (Theill *et al.*, 1989) is encoded by exons 1 and 2, encompassing amino acids 1−47 and 48−71. The activation domain can be divided into two subdomains (contained within amino acids 1−40 and 40−80), each of which is capable of conferring activation function upon the LexA DNA binding domain (Ingraham *et al.*, 1990). These subdomains, which we named STAα and STAβ, corresponding to exons 1 and 2, respectively, are separated by the 26 amino acid insert in GHF-2 (Figure 2B). Downstream to the STA domain GHF-1 contains a block of ~65 amino acids to which no function has been assigned. This region, named x, is encoded by exon 3 which also codes for the N-terminal portion of the POU-specific domain, characterized by a predominance of basically charged amino acid residues (Figure 2B; PSβ). While this part of the POU-specific domain is not essential for minimal DNA binding activity (Theill *et al.*, 1989) it is required for high affinity binding (Ingraham *et al.*, 1990). Interestingly, this module which is highly conserved among all POU-specific domains (Herr *et al.*, 1988), is also present at the 3′-end of a separate exon, exon 4, in the *Oct2* gene (Hatzopoulos *et al.*, 1990). The remainder of the POU-specific domain is composed of two potential α-helical sequences that are located entirely within exon 4 of *GHF1* and exon 5 of *Oct2* (Figure 2B; PSα). The POU homeodomain is encoded by exons 5 and 6. Like other homeodomains, it is composed of a short region rich in basic residues and three α-helical regions (Figure 2B: PHβ and PHα, respectively. In the *antennapedia* and *engrailed* homeodomains, the basic region reaches into and contacts the minor groove of the recognition sequence (Otting *et al.*, 1988; Kissinger *et al.*, 1990). In addition to its role in DNA binding, this region may also function in nuclear transport (Theill *et al.*, 1989). This region of the GHF-1 homeodomain is encoded by exon 5, whereas the bulk of the homeodomain including the three potential α helices, which compose the helix-turn-helix motif (Otting *et al.*, 1988; Kissinger *et al.*, 1990), is encoded by exon 6. In the majority of homeobox genes the entire homeodomain is encoded by one exon (Schneuwly *et al.*, 1986; Cho *et al.*, 1988; O'Connor *et al.*,

1988), although homeobox sequences split by an intron were also described (Poole *et al.*, 1985; Mlodzik *et al.*, 1988). The *GHF1* coding region outside the homeodomain is split by introns in phase I of the reading frame. This coincidence of phasing would permit exon shuffling. Taken together, our findings correlating GHF-1 protein structure and functional domains with *GHF1* genomic structure supports the hypothesis that gene segment duplication and exon shuffling (Rogers, 1985; Südhof *et al.*, 1985; Kirchgessner *et al.*, 1989) have contributed to the evolution of the POU subfamily of regulatory proteins.

Materials and methods

Isolation of cDNA clones

Rat GHF-2 cDNA clones were isolated from a rat GC cell cDNA library in λGT11 using the same methods previously used for isolation of GHF-1 cDNA clones (Bodner *et al.*, 1988). For PCR amplification the following two primers complimentary to the GHF-1 cDNA were used:

5′-AAGCTTGGATCCATGGGTTGCCAACCTTTCACCTCG-3′ and
5′-AAGCTTGCGGCCGCTACCACACATGGCTACCACAGG-3′.

Random primed first strand cDNA was prepared from 2 μg total RNA isolated from rat GC cells. 10% of the cDNA was amplified using standard procedures (Innis *et al.*, 1990). Amplified fragments in the size range of 0.9−1.0 kbp were isolated by low-melt agarose gel electrophoresis and cloned into the pBluescript IISK$^-$ vector. Clones containing rat GHF-2 sequences were selected by restriction site analysis. Twenty GHF-2 cDNA clones were identified and the complete DNA sequence of several clones were determined (Hattori and Sakaki, 1986).

Isolation of genomic GHF1 clones

1×10^6 plaque-forming units of a rat genomic library (Sargent *et al.*, 1979) were transferred to nitrocellulose filters and screened with GHF-1 cDNA probes (Bodner *et al.*, 1988). Positive plaques were identified after stringent washing (50°C, 0.1 × SSC, 50% formamide). DNA was purified from positive plaques and analyzed by restriction mapping (Maniatis *et al.*, 1982). DNA fragments from the genomic clones were subcloned into pBluescript SK$^-$ and sequenced using universal and gene-specific primers (Hattori and Sakaki, 1986).

Cell culture and transfections

RSVGHF2 was constructed by replacing the *Hin*dIII−*Not*I, GHF-1 coding fragment of RSVGHF1 (Theill *et al.*, 1989) by a similar fragment coding for GHF-2. pMTrGHF1α contains the human metallothionein II_A promoter (Karin and Richards, 1982) in front of the complete coding part (16.7 kbp) of the rat genomic *GHF1* gene cloned into pBluescript SK$^-$. pMGαG contains the $hMTII_A$ promoter in front of the rat *GHF1* gene exon 1, intron a, exon 2 plus part of intron b, followed by the human α-globin gene starting with intron a. pMGαG was constructed by insertion of a 4 kbp *Sma*I fragment from MTrGHF1α into $pUC\alpha_1$/SmaI (Yang-Yen *et al.*, 1990). The other plasmids used for the transfection experiments were described previously (Theill *et al.*, 1989; McCormick *et al.*, 1990). Rat6 cells were plated at 1×10^6 per 100 mm plate in DME containing 10% newborn calf serum. The following day, the cells were transfected with 5 μg of reporter plasmid plus a total of 20 μg of expression vectors containing RSV LTR. Cells were harvested 48 h later and assayed for CAT activity.

Western blot analysis

30 μg of crude nuclear extracts (Castrillo *et al.*, 1991) were separated by electrophoresis on a 12% SDS−polyacrylamide gel and transferred to nitrocellulose membrane. After transfer, the membrane was blocked using 2% dry milk and reacted with rabbit anti-GHF-1 antibodies (Bodner *et al.*, 1988) followed by goat anti-rabbit antibody coupled to horseradish peroxidase (Bio-Rad). The blots were developed using the chemilluminescence system (Amersham).

Protein preparations and DNA binding studies

*Nco*I−*Bam*HI fragments from RSVGHF1 and RSVGHF2 containing the coding regions of rat GHF-1 and GHF-2 cDNAs, were inserted into the T7 expression vector pET8C to generate pTGHF1 and pTGHF2. Proteins from these constructs were expressed in the bacterial strain BL21(DE3) and bacterial extracts were prepared and fractionated by 5−30% $(NH_4)_2SO_4$ cuts, followed by heparin−agarose chromatography as described (Buchan *et al.*, 1990). Further purification was achieved by FPLC MonoQ anion-exchange chromatography eluted with a 0−500 mM NaCl gradient in 10 mM potassium phosphate buffer pH 7.5, 0.1 mM EDTA, 10 mM DTT, 2% glycerol. The proteins were judged to be ~20% pure by Coomassie blue staining, as well as by Western analysis of SDS−polyacrylamide gels. The relative mobilities of bacterially expressed GHF-1 and GHF-2 on SDS−PAGE were essentially identical to those of the proteins expressed in Rat6 cells (Figure 6). GH_3 and GC whole cell extracts (WCE) were prepared as follows: 10^7 cells in monolayer were washed, pelleted and incubated in 5 pellet volumes of hypotonic buffer for 10 min at 4°C, followed by centrifugation and resuspension of the pellet in 1.5 vol of the same buffer. The cells were frozen in liquid N_2, thawed and the lysate made 0.4 N NaCl, rotated 30 min at 4°C and centrifuged 20 000 *g* 30 min to remove debris. Protease and phosphatase inhibitors were included. Gel retardation assays using recombinant GHF-1, GHF-2 or WCE were performed at 4°C for 20 min, in a 20 μl volume of 10 mM HEPES (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, 10% glycerol, 2.5 μg poly(dI·dC), 6 μg BSA, using 0.3 nM double-stranded rGH-P oligonucleotide, radiolabelled with T4 kinase. Free DNA and DNA−protein complexes were resolved at 4°C on a 6% non-denaturing polyacrylamide gel in 1 × Tris glycine buffer. Double stranded versions of the following oligonucleotides were used: rGH-P, 5′-AATTCCCATGAATAAATGTAT-AGGG-3′; mutGH, 5′-AATTCCCCTGGATCACTGGATCGGG; rGHF1-P, 5′-AGCTTACATGTATAAATGGATTTCCG-3′; rPRL-1P, 5′-AATTGATTATATATATATTCATGAA-3′.

RNase protection analysis

Total RNA isolation and RNase protection analysis was performed as previously described (Theill *et al.*, 1987). The plasmid pSK-520 consists of an *Eco*RI 530 bp rGHF2 fragment cloned into pBluescript SK$^-$. pSK-520 was linearized by *Not*I and the radioactively labelled antisense strand was synthesized by T3 polymerse. 25 000 c.p.m. of probe and 2−25 mg of total RNA were used.

In situ hybridization

Rat fetuses were prepared and *in situ* hybridization performed as previously described (Lazzaro *et al.*, 1991). The riboprobe was complementary to the first 68 bases of the GHF-2-specific insert.

Acknowledgements

We are grateful to Mark Bergseid at Stratagene Cloning Systems for help with GHF-2 protein purification. We thank Drs Yosef Shaul and Linda Haberstroh for helpful discussions; and Kathleen Sexton and Donna Caruso for help in preparing the manuscript. This study was supported by NIH grant DK38527 and post-doctoral fellowships from the Danish Natural Science Research Council (L.E.T.) and the California Division of the American Cancer Society (J.L.C.).

References

Baker,B.S. (1989) *Nature*, **340**, 521−530.
Behringer,R.R., Mathews,L.S., Palmiter,R.D. and Brinster,R.L. (1988) *Genes Dev.*, **2**, 453−461.
Berkowitz,L.A. and Gilman,M.Z. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5258−5262.
Bodner,M. and Karin,M. (1987) *Cell*, **50**, 267−275.
Bodner,M., Castrillo,J.-L., Theill,L.E., Deerinck,T., Ellisman,M. and Karin,M. (1988) *Cell*, **55**, 505−518.
Borrelli,E., Hayman,R.A., Arias,C., Sawchenko,P.E. and Evans,R.M. (1989) *Nature*, **339**, 538−541.
Buchman,C., Skroch,P., Dixon,W., Tullius,T.D. and Karin,M. (1990) *Mol. Cell. Biol.*, **10**, 4778−4787.
Castrillo,J.L., Theill,L.E. and Karin,M. (1991) *Science*, **253**, 197−199.
Chatelain,A. (1979) *Cell Tissues Res.*, **196**, 409−427.
Cho,K.W.Y., Goetz,J., Wright,C.V.E., Fritz,A., Hardwicke,J. and DeRobertis,E.M. (1988) *EMBO J.*, **7**, 2139−2149.
Crenshaw,E.B., Kalla,K., Simmons,D.M., Swanson,L.W. and Rosenfeld,M.G. (1989) *Genes Dev.*, **3**, 959−972.
Dollé,P., Castrillo,J.L., Theill,L.E., Deerinck,T., Ellisman,M. and Karin,M. (1990) *Cell*, **60**, 809−820.
Finney,M. and Ruvkin,G. (1990) *Cell*, **63**, 895−905.
Finney,M., Ruvkin,G. and Horvitz,H.R. (1988) *Cell*, **55**, 757−769.
Hattori,K. and Sakaki,Y. (1986) *Anal. Biochem.*, **152**, 232−238.
Hatzopoulos,A.K., Stoykova,A.S., Erselius,J.R., Goulding,M., Neuman,T. and Gruss,P. (1990) *Development*, **109**, 349−362.

Herr,W., Sturm,R.A., Clerc,R.G., Corcoran,L.M., Baltimore,D., Sharp,P.A., Ingraham,H.A., Rosenfeld,M.G., Finney,M., Ruvkin,G. and Horvitz,H.R. (1988) *Genes Dev.*, **2**, 1513–1516.
Hodin,R.A., Lazar,M.A., Wintman,B.I., Darling,D.S., Koenig,R.J., Larsen,P.R., Moore,D.D. and Chin,W.W. (1989) *Science*, **244**, 76–79.
Ingraham,H.A., Chen,R., Mangalam,H.J., Elsholz,H.P., Flynn,S.E., Lin,C.R., Simmons,D.M., Swanson,L. and Rosenfeld,M.G. (1988) *Cell*, **55**, 519–529.
Ingraham,H.A., Flynn,S.E., Voss,J.W., Albert,V.R., Kapiloff,M.S., Wilson,L. and Rosenfeld,M.G. (1990) *Cell*, **61**, 1021–1033.
Innis,M.A., Gelfand,D.H., Sninsky,J.J. and White,T.J. (eds) (1990) *PCR Protocols*. Academic Press, Inc., USA.
Izumo,S. and Mahdavi,V. (1988) *Nature*, **334**, 539–542.
Johnson,W.A. and Hirsch,J. (1990) *Nature*, **343**, 467–470.
Karin,M. and Richards,R. (1982) *Nature*, **299**, 797–802.
Karin,M., Castrillo,J.L. and Theill,L.E. (1990) *Trends Genet.*, **6**, 92–96.
Kirchgessner,T.G., Chuat,J.-C., Heinzmann,C., Etienne,J., Guilhot,S., Svenson,K., Ameis,D., Pilon,C., D'Auriol,L., Andalbi,A., Schotz,M.C., Gailbert,F. and Lusis,A.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9647–9651.
Kissinger,C.R., Liu,B., Martin-Bianco,E., Kornberg,T.B. and Pabo,C.O. (1990) *Cell*, **63**, 579–590.
Lambert,P.F., Spalholz,B.A. and Howley,P.M. (1987) *Cell*, **50**, 69–78.
Lazzaro,D., Price,M., DeFelice,M. and DiLauro,R. (1991) *Development*, **113**, 1093–1104.
Leroy,P., Krust,A., Zelent,A., Mendelsohn,C., Garnier,J.-M., Kastner,P., Dierich,A. and Chambon,P. (1991) *EMBO J.*, **10**, 59–69.
Li,S., Crenshaw,E.B., Rawson,E.J., Simmons,D.M., Swanson,L.W. and Rosenfeld,M.G. (1990) *Nature*, **347**, 528–533.
Lillie,J.W., Green,M. and Green,M. (1986) *Cell*, **46**, 1043–1051.
Maniatis,T., Sambrook,J. and Fritsch,E.F. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
McCormick,A., Brady,H., Theill,L.E. and Karin,M. (1990) *Nature*, **345**, 829–832.
Mermod,N., O'Neill,E., Kelly,T.J. and Tjian,R. (1989) *Cell*, **58**, 741–753.
Mlodzik,M., Fjose,A. and Gehring,J. (1988) *EMBO J.*, **7**, 2569–2578.
Mount,S.M. (1982) *Nucleic Acids Res.*, **10**, 459–472.
Nelson,C., Albert,V.R., Elsholtz,H.P., Lu,L. and Rosenfeld,M.G. (1988) *Science*, **239**, 1400–1405.
O'Connor,M.B., Binari,R., Perkins,L.A. and Bender,W. (1988) *EMBO J.*, **7**, 435–445.
Ohshima,Y. and Gotoh,Y. (1987) *J. Mol. Cell. Biol.*, **195**, 247–259.
Otting,G., Qian,Y.Q., Müller,M., Affolter,M., Gehring,W. and Wüthrich,K. (1988) *EMBO J.*, **17**, 4305–4309.
Poole,S., Kauvar,L.M., Drees,B. and Kornberg,T. (1985) *Cell*, **40**, 37–43.
Rogers,J. (1985) *Nature*, **315**, 458–459.
Rosner,M.H., DeSanto,R.J., Arnheiter,H. and Staudt,L.M. (1991) *Cell*, **64**, 1103–1110.
Sargent,T.D., Wu,J.-R., Sala-Trepat,J.M., Wallace,B., Reyes,A.A. and Bonner,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3256–3260.
Schneuwly,S., Kuriowa,A., Baumgartner,P. and Gehring,W.J. (1986) *EMBO J.*, **5**, 733–739.
Schöler,H.R., Dressler,G.R., Balling,R., Rohdewohld,H. and Gruss,P. (1990a) *EMBO J.*, **9**, 2185–2195.
Schöler,H.R., Ruppert,S., Suzuki,N., Chowdhury,K. and Gruss,P. (1990b) *Nature*, **344**, 435–439.
Simmon,D.M., Voss,J.W., Ingraham,H.A., Holloway,J.M., Briode,R.S., Rosenfeld,M.G. and Swanson,L.W. (1990) *Genes Dev.*, **4**, 695–711.
Slabaugh,M.B., Lieberman,M.E., Rutledge,J.J. and Gorski,J. (1982) *Endocrinology*, **110**, 1489–1497.
Südhof,T.C., Russell,D.W., Goldstein,J.L. and Brown,M.S. (1985) *Science*, **228**, 893–895.
Theill,L.E., Wiborg,O. and Vuust,J. (1987) *Mol. Cell. Biol.*, **7**, 4329–4336.
Theill,L.E., Castrillo,J.L. and Karin,M. (1989) *Nature*, **342**, 945–948.
Verrijzer,C.P., Kal,A.J. and van der Bliet,P.C. (1990) *EMBO J.*, **9**, 1883–1888.
Wirth,T., Priess,A., Annweiler,A., Zwilling,S. and Oeler,B. (1991) *Nucleic Acids Res.*, **19**, 43–51.
Yamamoto,K.K., Gonzalez,G.A., Menzel,P., Rivier,J. and Montminy,M. (1990) *Cell*, **60**, 611–617.
Yang-Yen,H.-F., Chiu,R. and Karin,M. (1990) *New Biol.*, **2**, 351–361.
Zelent,A., Mendelsohn,C., Kastner,P., Krust,A., Garnier,J.-M., Ruffenach,F., Leroy,P. and Chambon,P. (1991) *EMBO J.*, **10**, 71–81.

Received on October 21, 1991; revised on March 4, 1992