[B. Grandchamp *et al.*, *J. Biol. Chem.* **260**, 9630 (1985)]
14. Sources of plasmids used in this study: psp65Cl7N3 contains a human TGF-α cDNA insert as present in pTGF-C1 [R. Derynck *et al.*, *Cell* **38**, 287 (1984)]; pTGF-βas contains a 1.04-kb human TGF-β1 cDNA insert as present in λβCl [R. Derynck *et al.*, *Nature* **316**, 701 (1985)]; phEGF116 contains a 4.2-kb human EGF cDNA insert [G. I. Bell *et al.*, *Nucleic Acids. Res.* **14**, 8427 (1986)].
15. The following procedures were performed as described: acid-ethanol extraction of tissues [R. Derynck *et al.*, *Cancer Res.* **47**, 707 (1987)]; sandwich ELISA for TGF-α [T. S. Bringman, P. B. Lindquist, R. Derynck, *Cell* **48**, 429 (1987)]; NRK cell colony-forming bioassay for TGF-β [A. B. Roberts *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5494 (1980)]. In the radioimmunoassay for TGF-β1, a polyclonal goat antibody against recombinant human TGF-β1 and ^{125}I-labeled human TGF-β1 were used. The secondary antibody was an immunoglobulin G (IgG) fraction of serum obtained from a donkey immunized with goat IgG. The level of sensitivity was 400 pg/ml (R. Derynck, unpublished data).
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Purification of Growth Hormone–Specific Transcription Factor GHF-1 Containing Homeobox

JOSÉ-LUIS CASTRILLO, MORDECHAI BODNER, MICHAEL KARIN

Pituitary-specific expression of the growth hormone (GH) gene is governed by a transcription factor, GHF-1, that binds to two sites within its promoter. Recently, GHF-1 was shown to be a member of the homeobox family of DNA-binding proteins. An important question is whether GHF-1 controls the expression of other pituitary specific genes, such as prolactin (Prl), expressed in closely related cell types. To this end, GHF-1 was purified from extracts of GH- and Prl-expressing pituitary tumor cells and identified as a 33-kilodalton polypeptide. Although GHF-1 bound to and activated the GH promoter, it did not recognize the Prl promoter. However, at least one other factor in the same extracts, which was easily separated from GHF-1, bound to several sites within the Prl but not the GH promoter. Antibodies to GHF-1 did not react with the Prl binding activity. These results imply that the pituitary-specific expression of GH and Prl is governed by two distinct trans-acting factors.

THE PITUITARY-SPECIFIC EXPRESsion of the GH gene is due to the recognition of its promoter region by a specific transcription factor, GHF-1 (*1*). GHF-1 has, thus far, been detected only in GH-expressing cell types (*2–4*). Extinction of GH expression in somatic cell hybrids appears to be caused by repression of GHF-1 expression (*3*). However, when added to extracts of nonexpressing cells such as HeLa, GHF-1 activates the GH promoter (*1*). The analysis of recently isolated cDNA clones encoding GHF-1 has indicated that GHF-1 is a homeobox-containing protein (*4*) and therefore is a member of a large family of DNA-binding proteins that control development and differentiation (*5*). Immunohistological localization indicates that GHF-1 is expressed in cells of the somatotropic lineage in the anterior pituitary (*4*). GHF-1 may therefore be the major determinant specifying expression of GH in these cells.

An unresolved question is whether GHF-1 also controls expression of other anterior pituitary specific genes, such as prolactin (Prl). To address this question, we purified GHF-1 from whole cell extracts of pituitary tumor cells grown in suspension. These cells express both the GH and Prl genes (*6*).

A whole cell extract of GC cells (*7*) was fractionated by chromatography on heparin agarose and then analyzed on a Sephacryl S-300 gel filtration column (*8*). The GHF-1–enriched fractions were pooled and applied to a sequence-specific oligodeoxynucleotide-Sepharose column containing a high-affinity GHF-1 binding site (*9*). Purification of GHF-1 was monitored by deoxyribonuclease I (DNA I) footprinting. However, it was important to determine whether the transcriptional stimulatory activity of GHF-1 copurified with its DNA-binding activity. Furthermore, we wished to determine whether GHF-1 was the only pituitary cell–derived factor required for activating the GH promoter in extracts of nonexpressing HeLa cells. We therefore also monitored the transcriptional stimulatory activity of GHF-1 by adding a sample of each fraction to HeLa whole cell extract and measuring the level of transcripts initiated at the GH promoter by primer extension (*1, 10*). Each of the GHF-1–containing fractions stimulated initiation from the correct start site of the human GH (hGH) promoter in vitro, while having no effect on initiation from an adjacent nonspecific site (Fig. 1). Both start sites are used during transient expression of the hGH–chloramphenicol acetyltransferase (CAT) vector, although the physiological site is the dominant one (*2*). The ratio between the footprinting activity of GHF-1 (Table 1) and its ability to stimulate transcription was relatively unchanged during all three purification steps. Both activities eluted from the preparative S-300 column as a single peak corresponding to an apparent molecular mass of approximately 50 kD (*11*). A summary of the purification scheme is presented in Table 1.

SDS–polyacrylamide gel electrophoresis

Table 1. Purification of GHF-1.

Fraction	Protein (mg)	Volume (ml)	Units*	Yield (%)	Relative† purity
Whole cell extract	800	50	880,000	100	1
Heparin-agarose	250	30	750,000	85	3
S-300	27	54	450,000	51	17
DNA-affinity	0.21	10	150,000	17	700

*One footprinting unit is the amount of GHF-1 required for full protection of the proximal GH site with the use of a 5 ng of probe. †To determine relative purity, we assumed that the whole cell extract contained the same amount of GHF-1 as all of the heparin-agarose fractions, although it was never assayed directly by footprinting.

Department of Pharmacology, M-036, School of Medicine, University of California, San Diego, La Jolla, CA 92093.

(PAGE) of the affinity-purified GHF-1 indicated that it was still composed of several polypeptides (Fig. 2A). Further repetition of the affinity chromatography step did not result in a significant increase in the purity of GHF-1 even if the routinely used poly-(dI-dC) competitor was replaced with poly-(dA-dT). To identify which of the polypeptides corresponds to GHF-1, we subjected affinity-purified GHF-1 to preparative SDS-PAGE, and the polypeptides located within three regions of the gel were eluted, renatured (*12*), and examined by DNase I footprinting for GHF-1 binding activity. At least one of the three polypeptides that migrated between 34 and 32 kD bound to the hGH probe (Fig. 2B). In a subsequent experiment, each of these three polypeptides was separately eluted (Fig. 3) and renatured. Only the 33-kD species that migrated in the middle of the triplet exhibited significant binding to the hGH, indicating it is GHF-1 (Fig. 2C).

Although only one member of the 33-kD triplet had significant GHF-1 activity, the occurrence of these polypeptides at similar ratios in several different GHF-1 preparations and their similar molecular masses suggested that they might be structurally related. We therefore subjected them to peptide mapping with V8 protease (*13*). All three were composed of a common 19-kD peptide and a second smaller peptide, which added up to the size of the starting material. This raises the possibility that the 34- and 32.5-kD species, although structurally related to the 33-kD species, do not renature as readily after elution from the gel, which results in their failure to bind to the GH promoter.

The present data suggest that GHF-1 is the only pituitary-specific protein required for activation of the GH promoter in extracts of nonexpressing cells. Most important, the apparent molecular mass of the transcriptional stimulatory and DNA-binding activity determined by gel filtration (50 kD) is close to the molecular mass of the DNA-binding activity determined by SDS-PAGE (33 kD). These findings, and the specific localization of GHF-1 in vivo in cells of the somatotropic lineage, suggest that GHF-1 is the primary determinant of GH expression. Although GH-synthesizing (somatotropes) and Prl-synthesizing (lactotropes) cells are derived from a common precursor that expresses both genes (*14*), many of the cells in the adult pituitary express only one of the two (*15*). To determine whether the GH and Prl genes are both controlled by GHF-1 or by two distinct factors, we examined the binding of GHF-1 to the rat Prl (rPrl) promoter. Even though the rPrl probe was labeled to the same specific activity as the hGH probe, it was not significantly protected by GHF-1 (Fig. 4A). However, one or more factors present in the flow-through fraction of the affinity column protected five sites in the rPrl promoter. Four of these sites were identical to the ones previously assigned for the pituitary specific factor, Pit-1 (*16*), also known as LSF-1 (*17*). Since it was possible that the Prl-specific activity was a modified

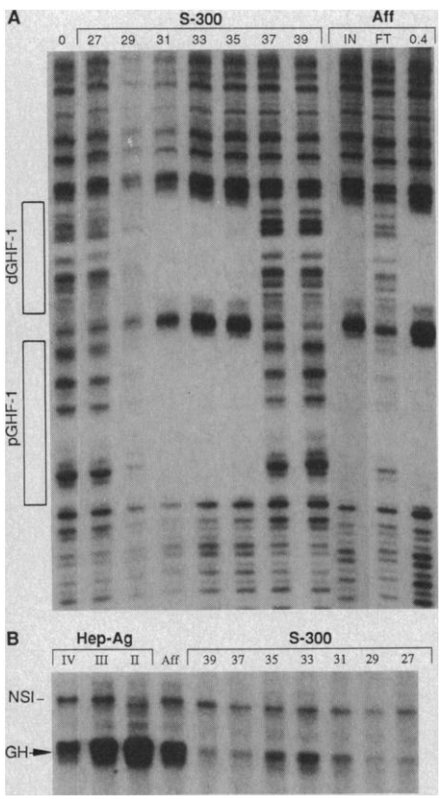

Fig. 1. Copurification of GHF-1 DNA-binding and transcriptional-stimulatory activities. A GC whole cell extract was first fractionated by heparin-agarose (Hep-Ag) chromatography. Hep-Ag fractions II and III were pooled and applied to a Sephacryl S-300 column. (**A**) Elution of GHF-1 was monitored by DNase I footprinting. The GHF-1–containing fractions were pooled and applied to a sequence-specific affinity column (Aff). The input (IN) and the eluate (0.4) contained GHF-1 binding activity, whereas the flow-through (FT) was depleted of this activity. (**B**) Transcriptional stimulatory activity of these fractions. A sample of each fraction was added to an in vitro transcription reaction mixture (HeLa whole cell extract and hGH-CAT template) (*1, 10*); the level of hGH-CAT transcripts was determined by primer extension (*1, 10*). The activity of GHF-1 was assessed by its ability to stimulate the formation of the correctly initiated hGH-CAT transcript (GH), while having no effect on the formation of an aberrant transcript initiated ten nucleotides further upstream (NSI). dGHF-1, distal GHF-1; pGHF-1, proximal GHF-1.

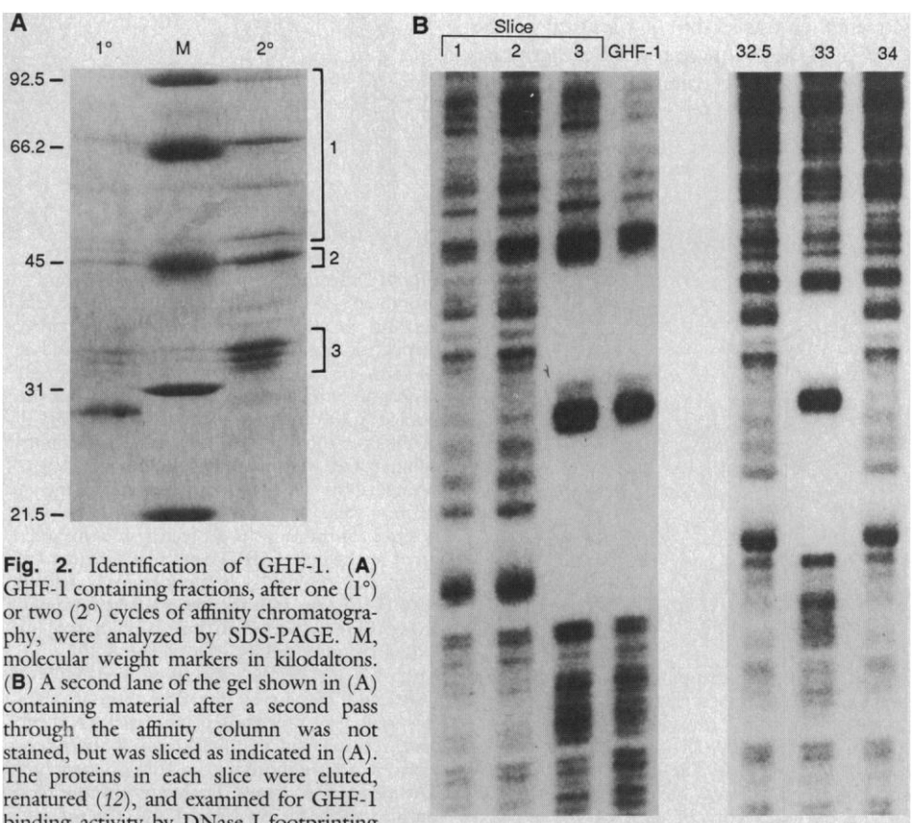

Fig. 2. Identification of GHF-1. (**A**) GHF-1 containing fractions, after one (1°) or two (2°) cycles of affinity chromatography, were analyzed by SDS-PAGE. M, molecular weight markers in kilodaltons. (**B**) A second lane of the gel shown in (A) containing material after a second pass through the affinity column was not stained, but was sliced as indicated in (A). The proteins in each slice were eluted, renatured (*12*), and examined for GHF-1 binding activity by DNase I footprinting analysis. In the lane labeled GHF-1, the footprints conferred by affinity-purified GHF-1 are shown for comparison. To further localize the GHF-1 binding activity, the three polypeptides migrating at 34, 33, and 32.5 kD were separated, eluted (Fig. 3), renatured, and examined for their ability to bind to the hGH promoter.

form of GHF-1 that no longer bound to the GH site, we used antibodies to GHF-1 to examine the relation between the two activities. These antibodies were generated against a synthetic peptide deduced from a partial sequence of the GHF-1 protein (*4*). These antibodies reacted strongly with the GHF-1 polypeptide in the S-300 and affinity-purified fractions but did not react with the flow-through fraction (Fig. 4B) that contained the Prl-specific binding activity.

These results differ from those of Nelson *et al.* (*16*), who reported that a single factor, Pit-1, binds to both Prl and GH. However, their conclusion was based on competition experiments and not on direct footprinting studies. Because of the similarity between the GH and Prl binding sites (Fig. 4C), a large excess of a GH site will probably compete for protection of the Prl sites. In fact, when moderate amounts of these sites were used, no cross-competition was observed (*18*). In addition, Siddiqui *et al.* (*17*) have found that unless it is first purified by a passage through a GH-specific affinity column, the Prl-specific factor LSF-1 is contaminated with GHF-1. Whereas these biochemical studies clearly support the existence of two separate factors controlling the GH and Prl genes, Ingraham *et al.* (*19*) have recently published the sequence of a cDNA claimed to encode Pit-1. Surprisingly, the sequence of this cDNA is identical to the GHF-1 cDNA sequence (*4*). Furthermore, although no direct binding of bacterially expressed Pit-1 to Prl was demonstrated, expression vectors containing the cDNA were capable of trans-activating the Prl promoter in a cotransfection assay (*19*). Since, at levels that are sufficient for full protection of both the high- and low-affinity sites of the GH promoter, GHF-1 does not significantly protect any of the previously assigned Pit-1 sites within the Prl promoter, including the proximal high-affinity site (Fig. 4), we suggest that the transactivation of Prl seen by the Pit-1 cDNA (identical in its sequence to GHF-1) is due to the vast overexpression of this protein in transiently transfected cells. When present in amounts far exceeding its physiological levels, it is possible that GHF-1 will bind not only to the GH promoter, but also to the structurally related Prl promoter. Interestingly, relaxed binding specificity was demonstrated for other homeobox proteins and appears to be a common property of this class of proteins (*20*). However, for proper regulation to occur in vivo, such proteins must interact with their targets in a highly specific manner.

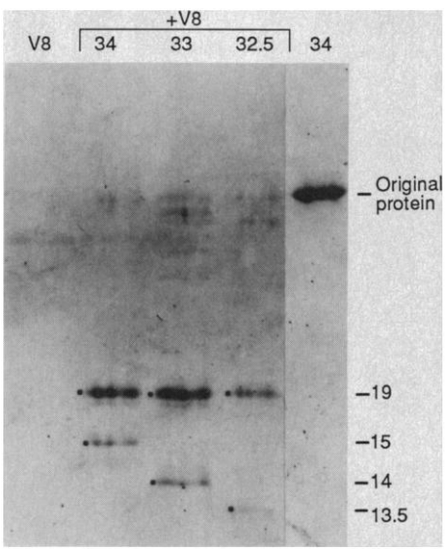

Fig. 3. V8 peptide mapping. To determine the structural relations between the three proteins migrating between 32.5 and 34 kD, they were separated by SDS-PAGE and subjected to V8 peptide mapping (*13*). The lane marked V8 contains V8 alone, whereas the lanes marked V8 + 34, 33, or 32.5 contain each of the isolated proteins plus V8. The lane marked 34 contains the original undigested 34-kD protein. The size of the resulting peptides are indicated on the side panel, in kilodaltons.

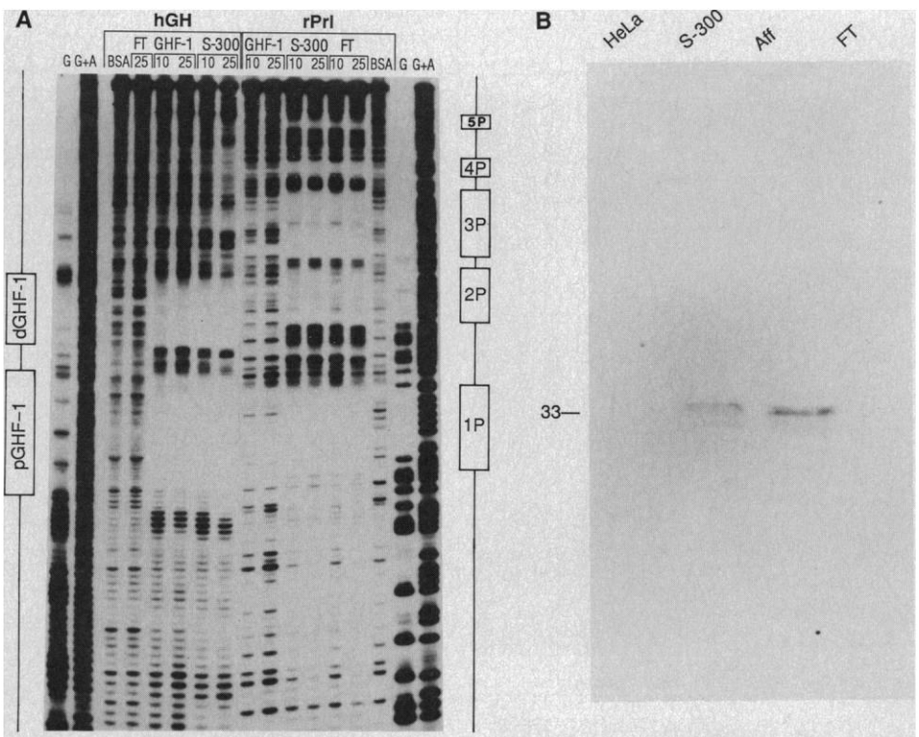

Fig. 4. Different factors bind to the GH and Prl promoters. (**A**) To determine whether the GH and Prl promoters are recognized by either distinct or similar factors, the hGH (labeled at position +3) and rPrl (labeled at position −8) probes were incubated with 10 or 25 μl of the pooled S-300 fraction, affinity-purified GHF-1 or the flow-through (FT) of the GHF-1 affinity column and were subjected to DNase I footprint analysis (*1*). The G and G + A sequencing ladders were used as size markers, and the position of the various protected sites are indicated by the boxes. (**B**) To examine whether the Prl-specific activity is immunologically related to GHF-1, the various protein fractions used above were subjected to immunoblot analysis with antibodies to GHF-1 (*4*). The S-300 (150 μg), 0.2 μg of the affinity-purified GHF-1 (Aff), and 150 μg of the flow-through (FT) fractions were precipitated with trichloroacetic acid, resuspended in SDS sample buffer, subjected to electrophoresis on a 10% polyacrylamide–SDS gel, and transferred to Immobilon membrane. The membranes were subjected to immunoblotting as described previously (*4*) with the use of affinity-purified antibodies to GHF-1 (*4*). The migration position of the 33-kD form of GHF-1 is indicated. (**C**) Sequence comparison of the various GHF-1 binding sites on the hGH and rGH promoters and the sites protected on the rPrl promoter. We have no proof whether the five Prl sites are recognized by a single factor; however, their sequences are quite similar and are shown only for comparison to the GHF-1 sites.

It will be of interest to isolate cDNA clones encoding the Prl-specific factor and determine whether this protein is also a homeobox-containing transcription factor. The analysis of the expression pattern of the GH- and Prl-specific factors during the development of the pituitary should help clarify how a single ectodermal stem cell leads to formation of an organ, the anterior pituitary, composed of many endocrine cell types expressing hormone genes.

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8. A whole cell extract of frozen GC cells was made as described previously (*1*), with slight modifications. Usually 35 g of packed cells were used. The protein pellets (800 mg of protein), obtained after the $(NH_4)_2SO_4$ precipitation step, were resuspended in 0.1*M* KCl, 20 m*M* Hepes (*p*H 7.9 at 30°C), 12.5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol (DTT), and 20% glycerol buffer (0.1*M* Hepes buffer). The resuspended proteins were dialyzed against 0.1*M* Hepes buffer, clarified by centrifugation, and applied slowly to a 25-ml heparin-agarose column (1 inch, or 2.5 cm, in diameter). The column was washed with 0.1*M* Hepes buffer and eluted with 0.4*M* KCl-Hepes buffer. Four 15-ml fractions were collected. Fractions 2 and 3 were pooled. Typically, they contained 80 to 90% of the GHF-1 activity, as monitored by DNase I footprinting. These fractions were supplemented with additional protease inhibitors: 0.5m*M* phenylmethylsulfonyl fluoride (PMSF), Pepstatin A, (1 mg/ml), Leupeptin (1 mg/ml), Aprotonin (1 mg/ml), and of soybean trypsin inhibitor (5 mg/ml) and applied to a 450-ml (1-inch diameter) column of Sephacryl S-300 (superfine), and equilibrated with 0.1*M* Hepes buffer containing 0.1% NP-40. The column was eluted with at least 500 ml of the same buffer and 9-ml fractions were collected. A portion of each fraction was removed for DNase I footprinting and the rest was frozen at −80°C. After identification of the GHF-1–containing fractions, they were thawed, supplemented with protease inhibitors as previously described, and applied to the affinity column.
9. The GHF-1 affinity column was prepared as described by J. T. Kadonaga and R. Tjian [*Proc. Natl. Acad. Sci. U.S.A.* **83**, 5889 (1986)] with the use of the following double stranded oligodeoxynucleotide, which conforms to the high-affinity GHF-1 binding site:

   5′-GATCCCATGCATAAATGTACACAG
   GGTACGTATTTACATGTGTCTTAA-5′

   The column contained 28 μg of DNA per milliliter of resin. Poly(dI-dC) was added at 1:100 ratio to the pooled S-300 fractions, which were split and loaded on 5 by 1 ml affinity columns at a rate of one drop per second. The columns were washed with 4 ml each of 0.1*M* Hepes buffer and eluted with 2 ml each of 0.4*M* Hepes buffer. The first 0.5-ml fractions were discarded and the remaining 1.5 ml of eluate, which contained GHF-1, were retained, pooled, and flash-frozen in liquid nitrogen. The protein was stored at −80°C. Approximate recovery of GHF-1 footprinting activity at this step was 30%.
10. DNase I footprinting reactions were performed as described (*1*). The hGH probe was a fragment from position +3 (labeled end) to −500. The rPrl probe was a fragment from position −8 (labeled end) to −400. In most instances, the fractionated protein preparations were diluted 1:100 in 0.1*M* Hepes buffer containing bovine serum albumin (BSA, 0.1 mg/ml). For in vitro transcription, undiluted samples (no more than 5 μl) of the various fractions were added to 20 μl of a HeLa whole cell extract and transcription reactions (total volume 50 μl, containing 200 ng of the hGH-CAT template) were performed and analyzed by primer extension (*1*).
11. The apparent molecular mass of GHF-1 was determined by its elution relative to molecular mass markers from the preparative S-300 column and an analytical S-200 column.
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13. Up to 2 μg of GHF-1 per lane were separated by electrophoresis on a 10% polyacrylamide–SDS gel and stained with Coomassie blue. The bands that correspond to the triplet around 33 kD were sliced and inserted into the wells of a second 15% polyacrylamide (0.4% bisacrylamide)–SDS gel. The slices were coarsely chopped while inserted and loading buffer was added, consisting of V8 protease (5 ng/ml, from *Staphylococcus aureus*), 1% SDS, 10 m*M* K_3PO_4 (*p*H 8.0), 10% glycerol, 20 m*M* DTT, and 0.05% bromophenol blue. The gel slices were soaked in the loading buffer for 10 min, separated by electrophoresis for 30 s, and incubated for another 15 min. Then electrophoresis was reinitiated and allowed to proceed for 90 min until the bromophenol blue dye reached the bottom. The gel was stained with Coomassie blue.
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