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A "20K" Form of Growth Hormone in the Murine Pituitary Gland[1] (41974)

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Abstract. Extracts of mouse and rat adenohypophyses have been analyzed for a low-molecular-weight variant of growth hormone (GH) known to occur in humans, the so-called "20K"-GH (mol wt = 20,000 vs 22,000 for traditional human GH). Pituitary proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunostained with an antiserum raised against murine GH. Reactive and unreactive bands in close vicinity of the major GH band were fingerprinted by a peptide-mapping technique that reveals only the tyrosine-containing peptides, but can be applied to fingerprint single protein bands within gels. We found a protein band 2000 mol wt smaller than the major murine GH in both mouse and rat pituitary glands whose fingerprint resembled that of major GH. The peptide-mapping results are consistent with the interpretation that the internal deletion of amino acid residues most likely is in the same region of the molecule as in the human 20K-GH. Unlike the human 20K-GH, however, the murine counterpart showed no cross-reactivity with an antiserum raised against 22K-GH in the test system used here.

The pituitary growth hormone (GH) was discovered in the early 1920s and was so-named because of its ability to stimulate somatic growth. However, many other actions besides growth promotion have since been found for this substance ranging from stimulation of protein, carbohydrate, and fat metabolism to mammotropic and renotropic effects. Oddly enough, several of these actions are contradictory. For example, both an insulin-like effect and an anti-insulin hyperglycemic effect as well as a lipolytic and an antilipolytic effect have been reported for human GH (1). Such observations have led to the notion that GH is perhaps a complex of proteins whose members each express different functions (2). The serious discrepancies found between the bioassay and radioimmunoassay measurements of GH strengthen this concept (3). Furthermore, stimuli such as insulin-induced hypoglycemia, arginine, dopamine, or stress, which cause prompt and marked release of radioimmunoassayable GH in primates, completely fail to do so in laboratory animals (4).

Several recent advances, both in the area of GH purification and cloning of the GH gene, indicate that there indeed may be different molecular forms of GH. For example, a variant of human GH has been isolated (5, 6) in which a chain of 15 amino acids (residues 32–46) is missing (7). It is generally referred to as 20K-hGH, because its molecular weight is 2000 smaller than the traditional hormone, which is referred to as 22K-hGH. The variant, 20K-hGH, has growth-promoting activity similar to that of human 22K-GH (5), but lacks the early insulin-like (8) and hyperglycemic (9) effects ascribed to human GH. Furthermore, 20K-hGH is only one-third as active as the 22K-hGH in the radioimmunoassay (5) and seems to have altered receptor-binding characteristics (10, 11). Structural analysis disclosed that the human GH gene contains four intervening sequences or introns and that the 20K-hGH molecule results from the alternate splicing of the human GH mRNA precursor at a position 45 nucleotides downstream from the 3 in. end of the B intron (12). Significantly, the rat GH gene also possesses four introns, with the B intron occurring in the same position as the corresponding intron in the human GH gene (12). Thus, there is a high probability that a variant such as, or similar to, 20K-hGH may also exist in this species. The extremely small size of pituitary glands in rats and mice presents serious difficulties

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in searching for such an entity by conventional means. However, use of sensitive analytical tools has enabled us to detect a smaller molecular weight variant of murine GH, as described.

Experimental Procedures. *Animals and tissues.* Mice and rats were purchased from the Simonsen laboratory, Gilroy, California. The inbred strains such as the C3H/St and C57BL/6J mice were obtained from Scripps Clinic and Research Foundation, La Jolla, California. All animals were housed in air-conditioned (70 ± 1°C), light-controlled (12 hr light:12 hr dark) quarters and were given Wayne Lab Blox and tap water *ad libitum.* Their pituitary glands were removed after decapitation, and the posterior lobes were separated and discarded. The anterior lobes were washed three to four times with cold saline (0.85% NaCl) to remove blood. Each lobe was then homogenized immediately either in 0.05 *M* Na_2CO_3–$NaHCO_3$ buffer, pH 10, in the ratio of 10 mg/ml or in Laemmli's (13) sample buffer (0.0625 *M* Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromphenol blue, with or without 5% 2-mercaptoethanol) in the ratio of 20 mg/ml. Samples with 2-mercaptoethanol were heated in a boiling water bath for 5 min. All homogenates were centrifuged at 1000*g* for 15 min and the supernatants were used for electrophoresis.

Gel electrophoresis. Pituitary proteins were separated by polyacrylamide gel electrophoresis (PAGE) with or without $NaDodSO_4$. Gel electrophoresis with $NaDodSO_4$ was carried out in cylindrical gels by the procedure of Weber and Osborn (14) and in slab gels by using the buffer system of Laemmli (13). The cylindrical gel consisted of 10% acrylamide and was 0.5 cm in diameter and 10 cm in length; the slab gel consisted of 12% acrylamide and was 12.5 cm long (separating part) and 1.5 mm thick. Gel electrophoresis without $NaDodSO_4$ was carried out at pH 8.3–8.5 by the method of Davis (15). This gel consisted of 10% acrylamide and was 0.5 cm in diameter and 7.5 cm long. Twenty-five microliters of the pituitary extract made in Laemmli's sample buffer (0.5-mg equivalents of tissue) was applied in gels with $NaDodSO_4$ and 100 μl of the pH 10 extract (1-mg equivalents of tissue) in gels without $NaDodSO_4$. $NaDodSO_4$ gels were stained with Coomassie brilliant blue R-250 and destained by diffusion in 5% methanol and 7.5% acetic acid. Gels without $NaDodSO_4$ were stained with Coomassie blue G-250 and destained by diffusion in water.

Electroblotting. Immediately after the completion of electrophoresis, proteins from some slab gels were transferred onto nitrocellulose paper as outlined by Towbin *et al.* (16) by using Bio-Rad's electroblotting equipment. The electroblotting was performed for 3.5 hr. After transfer, the paper was air-dried at 4°C.

Immunostaining. Existence of GH-like proteins in the pituitary gland was probed further by staining the pituitary proteins in the gel or in the nitrocellulose paper with a monkey anti-mouse GH serum. The method is an adaptation of the procedure described by Adair *et al.* (17). The anit-mouse GH serum used was prepared in our laboratory by injecting a highly pure preparation of mouse GH into rhesus monkeys (contaminating proteins constituted less than 1% of total as determined by electrophoresis). The antiserum is highly specific for GH; other known pituitary hormones do not cross-react with it. Details regarding immunization and specificity are given in earlier publications (4, 18). Further results on specificity are presented in the results section. Cylindrical gels, or lanes from slab gels, were sliced transversely with a gel slicing device before immunostaining. Slicing allowed the antibody molecules, which could not enter these 10 and 12% gels, to bind to proteins exposed on the surface of the gel. The sliced gel or the nitrocellulose paper was then incubated with a 1:50 dilution of anti-mouse GH serum for desired intervals. The unbound antibody was washed off and the immunostained gel or paper was reacted with $\sim 2 \times 10^6$ cpm/ml of ^{125}I-labeled Protein A (New England Nuclear, Boston, Mass.). The reactive bands were visualized by autoradiography.

Peptide mapping. For structural comparisons of proteins suspected to be related to GH, we used the peptide mapping method of Elder *et al.* (19), which requires less than a microgram of protein. This method reveals only peptides that contain tyrosine residues, but the patterns are quite characteristic for a

given protein. Briefly, protein bands to be mapped were excised from the gel and diced into 1- to 2-mm cubes. The gel fragments were washed exhaustively in 10% methanol to remove $NaDodSO_4$ and other contaminants. The washed fragments were dried under a heat lamp and then iodinated by sequential addition of 20 μl of 0.5 *M* sodium phosphate buffer, pH 7.5, 0.50—1.0 mCi of ^{125}I (5 μl) and 5 μg of chloramine T (5 μl). After incubation for 30 min, the reaction was stopped by adding 2 mg of sodium metabisulfite in 1 ml of water, and incubation continued for 1 hr. The labeled gel fragments were then washed exhaustively with 10% methanol to remove free ^{125}I. Next, they were dried again in preparation for digestion of the iodinated protein, which was accomplished by adding 150 μl of a solution of TPCK-trypsin (Worthington Biochemicals, Freehold, N.J.) in 0.05 *M* ammonium bicarbonate, pH 8.0, and incubating at 37°C for 4–6 hr. The tryptic peptides were eluted by adding 0.5 ml of distilled water and incubating further for 18 hr. The supernatant containing tryptic peptides was removed and lyophilized. The lyophilized digest was dissolved in a few microliters of high voltage electrophoresis buffer (acetic acid:formic acid:water = 15:5:80), and a 5- to 10-μl sample containing approximately 10^6 cpm was spotted on a 10 × 10-cm cellulose-coated glass TLC plate (EM Laboratories, Elmsford, N.Y.). The digest was analyzed in two dimensions: by thin-layer electrophoresis in the first dimension followed by ascending chromatography in the second dimension. The chromatography buffer consisted of butanol:pyridine:acetic acid:water (32.5:25:5:20). Plates were dried at room temperature and the peptides were visualized by autoradiography on Kodak XAR X-ray film.

Autoradiography. The gels were dried by soaking in 70% methanol for 20 min, placement on thick filter paper and application of a vacuum (0.5 μm) and heat (80–82°C) for 2 hr in a commercial slab drier (Bio-Rad, Richmond, Calif.; Model 224). Dried gels, nitrocellulose papers, or TLC plates were exposed to Kodak XAR films and a DuPont Hi-Plus intensifying screen for appropriate intervals. Films were developed in a Kodak RP X-Omat processor.

Results. Fig. 1 shows the degree of separation between pituitary proteins from mice and rats by three electrophoretic methods

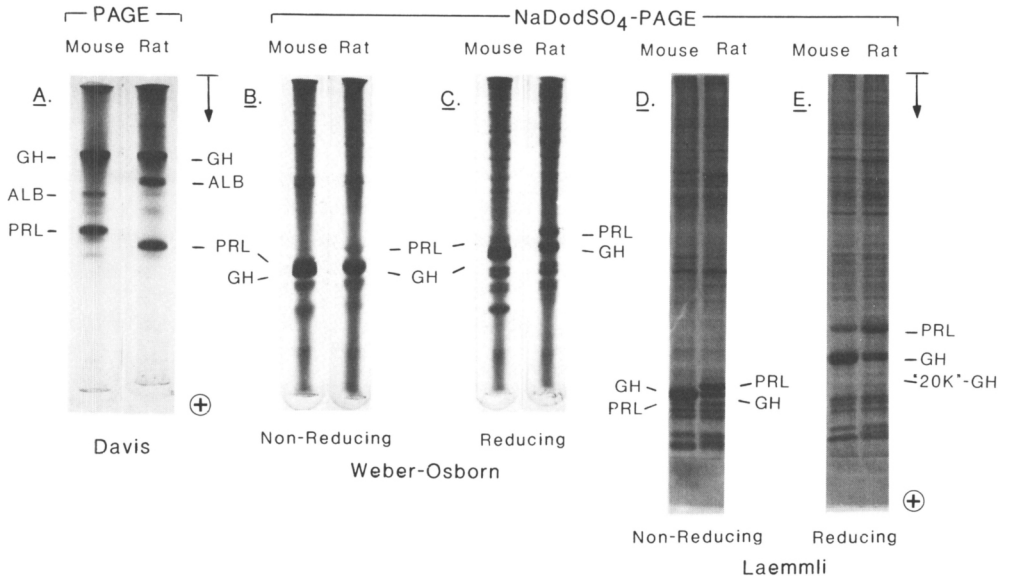

FIG. 1. Electrophoretograms showing the degree of separation of murine pituitary proteins by three different electrophoretic methods described under Materials and Methods. A, B, and C, cylindrical gels; D and E, slab gels. Extract of 1 mg of anterior pituitary tissue was used in A and of 0.5 mg in B–E.

[Laemmli (13), Weber and Osborn (14), and Davis (15)]. The positions of the main GH and PRL bands were established by concurrently running purified preparations of these hormones. GH and PRL appeared to be two of the major proteins in the murine pituitary gland by all three methods. In PAGE, separation is by size as well as charge; therefore, the GH band was widely separated from the PRL band. In $NaDodSO_4$–PAGE, separation is by size alone, and since the molecular weight of GH and PRL are very close (22,000 vs 23,000, respectively), they migrated very near to one another. The GH molecule has two disulfide bridges and the PRL molecule, three. Adding 2-mercaptoethanol in the $NaDodSO_4$ gel reduces these disulfide bonds and thereby increases the Stokes radius of these molecules. Therefore, in such gels (C and E), the migrations of both hormones were further retarded and the separation between the two was increased. By far, the best separation of the different pituitary proteins, particularly that of GH and PRL, was achieved in slab gels run under reducing conditions (E). Furthermore, the Laemmli system exposed several minor protein bands not seen in Davis or Weber–Osborn gels.

We then looked at the electrophoretic mobility of 22K-hGH and 20K-hGH in a slab gel electrophoresed under both reducing and nonreducing conditions. The 20K-hGH migrated ahead of the standard hGH and was distinctly separate from the latter (data not shown). From this characteristic, we reasoned that if there were a murine "20K"-GH, it would migrate just ahead of the main GH band in the $NaDodSO_4$ gels, approximating the position of the band so-marked in Fig. 1E, in the case of slab gels.

We next examined proteins in murine pituitary extracts for cross-reactivity with GH antibodies by immunostaining gels directly with antiserum to mouse GH using cylindrical gels with or without $NaDodSO_4$. Figure 2A shows that only the GH band stained with this antiserum; even after 16 hr of film exposure, the PRL band or other protein bands failed to show any cross-reactivity, confirming that the reaction was specific for GH-related proteins. In Fig. 2B, we see that after 2 hr of film exposure, the major GH band showed reactivity, as did the area just behind it, which encompassed the PRL band. However, the PRL band in PAGE without $NaDodSO_4$ (A) and standard PRL in $NaDodSO_4$–PAGE under reducing conditions (data not shown) were completely unreactive. These results rule out PRL as the source of immunoreactivity in the area immediately behind the GH band and suggest that a GH variant slightly larger in molecular weight

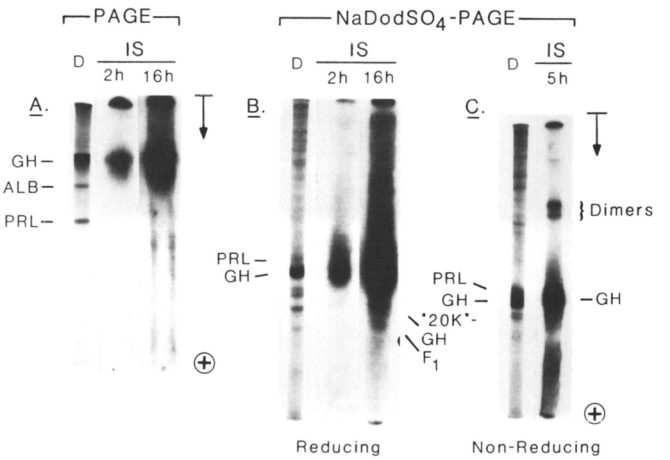

FIG. 2. Immunological cross-reactivity of *mouse* anterior pituitary proteins, separated in cylindrical gels with or without $NaDodSO_4$, with antibodies against mouse GH. Extract of 1 mg of anterior pituitary tissue was used in A and of 0.5 mg in B and C. A 1:50 dilution of a monkey antiserum to mouse GH was used for the immunostaining. D = dye-stained gel; IS = immunostained gel after 2 or 16 hr of film exposure. Only one exposure of 5 hr is shown in C.

than the major murine GH may exist. When the film was exposed for 16 hr, two additional bands of low molecular weight appeared—one just ahead of the major GH band and another further ahead. From its R_f, the first of the two low molecular weight bands was a likely candidate for "20K" murine GH. The second low-molecular-weight band most likely represented the F_1 fragment of cleaved GH (20), as ascertained from comparison of its R_f with that of cleaved GH in our standard mouse GH preparation. Under nonreducing conditions (C), two closely situated immunoreactive bands of high molecular weight appeared in the area where dimers of GH are expected to migrate. The lower of the two bands could represent dimers of low-molecular-weight immunoreactive components seen in Fig. 4. Virtually identical results were obtained in experiments with rat pituitary glands (Fig. 3).

The immunostaining reaction of the tentatively designated "20K"-GH band in Figs. 2 and 3 was quite weak, although this region of the Weber–Osborn gel contained several major bands, as revealed by dye staining (Fig. 1C). This raised the possibility that "20K"-GH was not one of the heavily staining bands but rather it was a minor component comigrating with these prominently staining substances. A way to separate such a minor protein from the prominent bands in the region was thus needed. Pituitary extracts were electrophoresed in slab gels for this purpose. As seen in Fig. 1E, the two heavy bands ahead of the GH band pulled further away from the latter and several new, minor bands emerged in the area vacated.

The results of immunostaining such a gel are shown in Fig. 4. The proteins were transferred from the gel onto the NTC paper by electroblotting in this experiment. As expected, the main GH band stained heavily in both nonreducing and reducing gels. A faint band behind GH, perhaps representing a glycosylated form of GH such as that found for PRL (26), was also visible (B). The band designated "20K"-GH, on the other hand, showed very little, if any, immunoreactivity in this gel (B). However, several other minor bands ahead of the main GH band cross-reacted, in both the mouse and rat pituitary extracts. The identity of the first three remains unknown; the fourth and fastest migrating most likely represented the *N*-terminus fragment of cleaved GH (2), as judged from comparison of its R_f with that of cleaved GH in our standard mouse GH preparation (data not presented). In the nonreduced state (A), several bands of high molecular weight reacted with the antibody, all of which disappeared when the pituitary extract was electrophoresed under reducing conditions. These results indicated that the band designated "20K"-GH in tube gels (B in Figs. 2 and 3) was not a single protein but indeed a mixture of several different proteins including "20K"-GH.

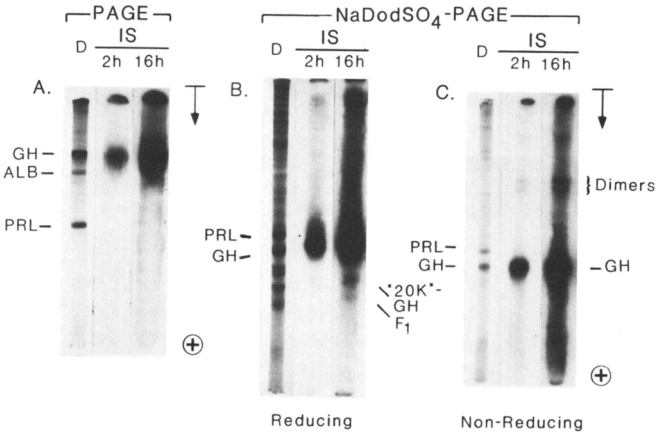

FIG. 3. Immunological cross-reactivity of *rat* anterior pituitary proteins, separated in cylindrical gels with or without $NaDodSO_4$, with antibodies against mouse GH. For other details, see legend to Fig. 3.

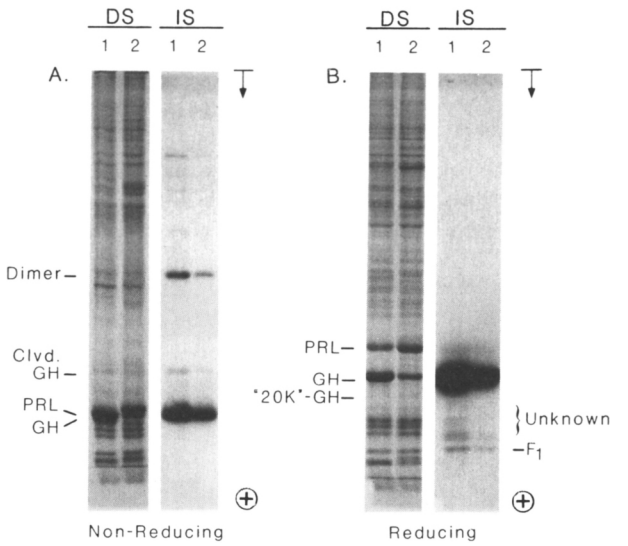

FIG. 4. Immunological cross-reactivity of murine anterior pituitary proteins, separated in a slab gel and electroblotted onto nitrocellulose paper, with antibodies raised against mouse GH. Extracts of 0.5 mg of anterior pituitary tissue were used in each case. A 1:1000 dilution of a monkey antiserum to mouse GH was used for the immunostaining. DS = dye-stained gel; IS = immunostained gel after 30 hr of film exposure. Lane 1 = mouse; Lane 2 = rat.

Figure 5 shows the amino acid sequence of rat GH molecule, derived from the GH gene structure (21), and the points of tryptic cleavage. There are seven tyrosine residues in the molecule. Thus, a tryptic digest of iodinated rat GH will have seven labeled fragments and a tryptic peptide map of the hormone will be expected to elicit seven major radioactive spots. The complete amino acid sequence of mouse GH has not been determined, but is believed to be similar to that of rat GH (22). From the known structures of 22K-hGH and 20K-hGH, such a peptide mapping of these proteins should yield five and four spots, respectively. If the putative murine "20K"-GH has amino acids deletion in the same region and of the same magnitude as in 20K-hGH, its tyrosine–peptide map would have *two* fewer spots than the main murine GH.

A number of protein bands from the regions of the tube and slab gels suspected of having "20K"-GH were peptide mapped. Figure 6 presents the result for a few of the protein bands from slab gels electrophoresed under reducing conditions, such as that shown in Figs. 1E or 4B. Both mouse and rat GH standards (A and D) elicited seven major spots and a few minor ones, presumably from contaminants, their patterns being virtually identical. Mouse and rat PRL standards (I and L) elicited patterns very similar to one another, each consisting of six major spots, as predicted from the known structure of rat PRL (23), and the patterns were quite distinct from those of murine GH. The peptide maps of hGH (B) and hPRL (F) standards were also different from one another and different from those of their murine counterparts. Peptide maps of the GH band in crude mouse (G), rat (J), and human (E) pituitary extracts produced patterns similar to those of the purified standards. The peptide map of the 20K-hGH standard (C) was virtually identical to that of 22K-hGH standard (B) or the human GH band from the pituitary extract (E), with the exception that, as predicted from its structure, one major spot was missing (indicated by the circle). Peptide maps of the bands designated "20K"-GH in Figs. 1E or 4B, both in the case of mouse and rat pituitary extracts (H and K), also resembled those of the mouse and rat GH standard preparations (A and D) as well as gel-derived materials (G and J), except that two major spots (indicated by circles) were

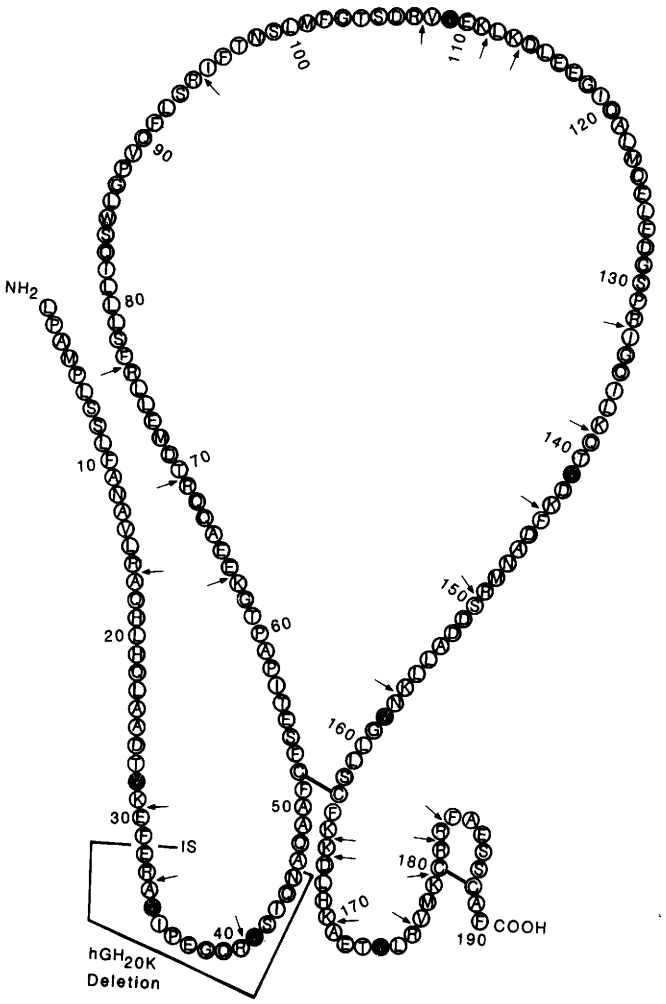

FIG. 5. Diagram showing the amino acid sequence of a rat GH molecule. The sequence is based upon the structure of rat GH gene (21). One-letter symbols designate amino acids. The dark circles represent tyrosine residues. Arrows indicate the points of tryptic cleavage. The region of deletion in the human 20K-GH is indicated at the bottom, left. IS = intervening sequence.

missing. These results confirmed the identity of the "20K"-GH band as a small molecular weight variant of murine GH. Peptide maps of the other bands in the area, designated as "unknown" in Fig. 4B, did not bear an overt resemblance to that of murine GH (data not presented).

Discussion. Although 20K-GH was discovered in humans nearly a decade ago (24), its existence in other species has not been documented until now. In the present study, we employed newly developed, sensitive techniques for its detection in the mouse and rat pituitary gland, where the supply of tissue to work with is meager. The results obtained show that a "20K" form of GH does exist in mouse and rat pituitary glands. (We use the term "20K"-GH here only for the purposes of conformity with the literature; our estimates of the molecular weight of mouse GH from its R_f in nonreducing slab gels consisting of 10% acrylamide give a value of only 19,500 (25), and thus the molecular weight of mouse "20K"-GH would be approximately 17,500.) The glandular concentration of murine "20K"-GH, however, ap-

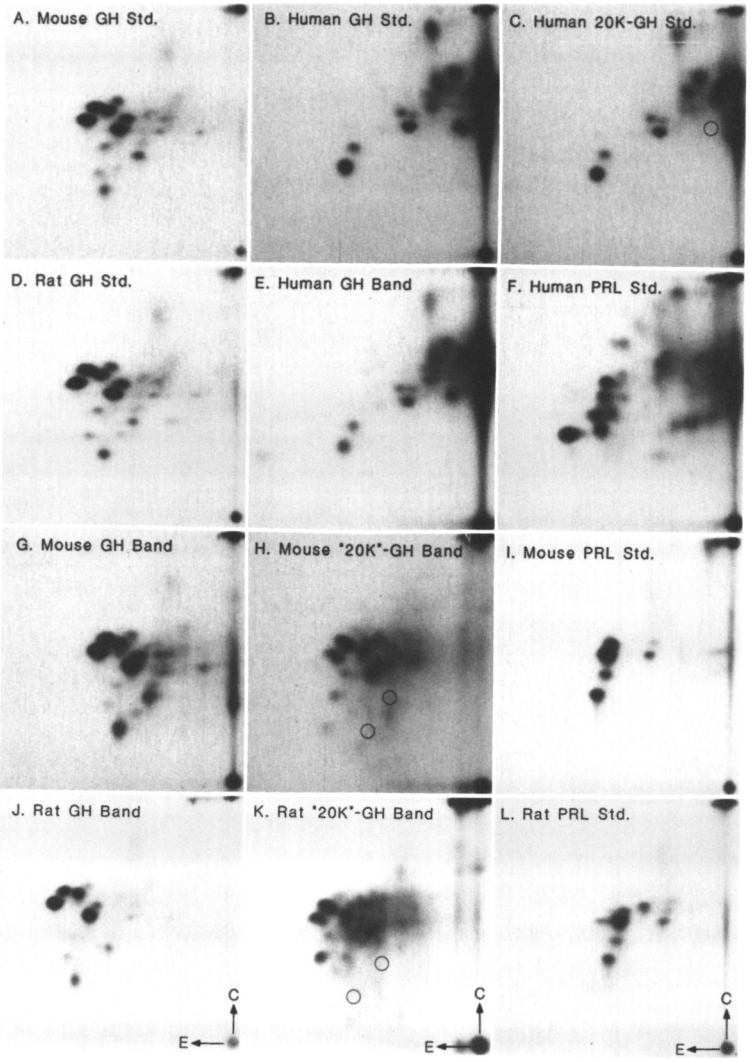

FIG. 6. Partial peptide maps of the murine "20K" bands shown in Fig. 1E and some known standards. Only tyrosine-containing peptides are visible. Circles indicate the positions of the missing peptides. Arrows indicate the directions of electrophoresis (E) and chromatography (C).

pears rather low: in mice it constituted <1% and in rats 1–2% of total GH. In contrast, 20K-hGH forms nearly 5–10% of total hGH (5).

Our peptide-mapping results are consistent with the interpretation that the deletion of amino acids in murine "20K"-GH is most likely in the same region as in human 20K-GH. In the latter, residues 32–46 are absent from a chain of 191 amino acids (7). As shown in Fig. 6, deletion of residues 32–46 in the rat GH molecule would result in loss of two tyrosine residues, and a tryptic digest would have two fragments each with a tyrosine residue missing. A tryptic peptide map of murine "20K"-GH, based on the iodination of tyrosines, would therefore have two fewer spots. Indeed, in the peptide maps of "20K"-GH bands from both mouse and rat pituitary glands two major spots were absent, confirming that two tyrosine-containing peptides were missing. However, it should be recognized that deletions in other regions of the molecule could also elicit such a finger-

print. Complete peptide map and amino acid sequence analysis of purified material is needed to confirm the site and extent of deletion. Among the five remaining major spots, the migrations of the two differed somewhat in material from a mouse and a rat. This implies that the amino acid sequences of mouse and rat GH in the regions represented by these two peptides might be different. Colosi and Talamantes (22) have sequenced the first 10 *N*-terminal residues of mouse GH, and found a difference at position 1 (and possibly at 8) from the rat GH sequence.

Our results suggest that the alternate splicing of human GH pre-mRNA that gives rise to 20K-GH (12) is not limited to humans but occurs in other species as well. Although differences in the actions of 22K-hGH and 20K-hGH have been reported (8, 9), the primary physiological function of 20K-GH is unknown. Demonstration of "20K"-GH in laboratory animals and availability of techniques for its detection should facilitate studies of the physiology of this GH variant.

The existence of additional variants and fragments of GH is also suggested by these studies. Both an area just behind the major GH band in Figs. 2B, 3B, and 4B, and an area ahead of "20K"-GH in Fig. 4B, designated unknown and containing several minor bands, exhibited immunoreactivity. However, in view of the heterogeneity of the polyclonal antibody used here, their relatedness to GH remains to be established. Availability in the future of monoclonal antibodies to murine GH may provide confirmation of these findings. The substantial amounts of contaminating proteins in these regions have so far made it difficult to obtain an unequivocal peptide map. Nevertheless, it is probable that the high molecular weight component represents a glycosylated form of GH, similar to that found for PRL (26). Whether the low M_r components represent fragments of GH or are independent entities remains unknown. They migrated behind the F_1 fragment of cleaved GH, which distinguishes them from cleaved GH found in purified preparations. Efforts are under way to separate these by two-dimensional electrophoresis for further characterization.

The physiological significance of murine 20K-GH can only be conjectured at this time. Unlike the human counterpart (5), it showed complete lack of immunological cross-reactivity in the test system used here. If it is found to circulate in blood, as the human counterpart has recently been found to do (27, 28), its secretion could account for some of the unexplained observations of radioimmunoassays (3, 4).

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