Primary Sequence of Two Regions of Mouse Pro-adrenocorticotropin/Endorphin†

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ABSTRACT: The amino acid sequences of two previously uncharacterized regions of the mouse anterior pituitary common precursor to adrenocorticotropin (ACTH) and β-endorphin (pro-ACTH/endorphin) were determined. Portions of the NH_2-terminal region of pro-ACTH/endorphin (called the 16K fragment) and the region between ACTH and β-endorphin (called γ-lipotropin) were sequenced by Edman degradations of biosynthetically labeled immunoprecipitated proteins and by Edman degradations of purified 16K fragment and β-lipotropin. With a combination of these two approaches, 29 of the first 34 residues at the NH_2-terminal end of the mouse 16K fragment were determined. The NH_2-terminal region of the mouse 16K fragment was found to be nearly identical with the homologous porcine and bovine molecules. The complete amino acid sequence of the NH_2-terminal region of γ-lipotropin was determined. In contrast to the highly conserved nature of the 16K fragment, mouse γ-lipotropin was found to differ substantially from the γ-lipotropins of other species. Although the NH_2-terminal and β-melanotropin-like regions of mouse γ-lipotropin are similar to the corresponding regions of other γ-lipotropins, the intervening region of mouse γ-lipotropin is substantially shorter than it is in other γ-lipotropins. In addition, mouse γ-lipotropin lacks the pair of basic amino acids that normally mark the proteolytic cleavage site used to produce β-melanotropin from γ-lipotropin.

Studies of pituitary tissue from a number of species have established that a single molecule serves as the biosynthetic precursor to adrenocorticotropin (ACTH),[1] β-lipotropin (βLPH), 16K fragment (the glycopeptide comprising the NH_2-terminal region of pro-ACTH/endorphin), and related smaller peptides (Eipper & Mains, 1980a; Loh, 1979; Chretien et al., 1979; Herbert et al., 1980; Cohen et al., 1980) (Figure 1). Selective proteolytic cleavage of pro-ACTH/endorphin leads to production of the peptides characteristic of the anterior pituitary (primarily ACTH, βLPH, and 16K fragment) or the intermediate pituitary [primarily α-melanotropin [αMSH; *N*-acetyl-ACTH(1–13)NH_2], corticotropin-like intermediate lobe peptide [ACTH(18–39)], γLPH [and in some species N fragment; in bovine, γLPH(1–40)] plus β-melanotropin [βMSH; in bovine, γLPH(43–60)], β-endorphin, and 16K fragment and related smaller peptides] [reviewed by Jackson & Lowry (1980), Eipper & Mains (1980a), Krieger et al. (1980), and Bradbury et al. (1976)].

The complete amino acid sequence of bovine intermediate pituitary pro-ACTH/endorphin has been predicted from nucleotide sequencing of the ribosomal precursor by Nakanishi et al. (1979). The overall length of the bovine molecule is 239 amino acids, punctuated by ten pairs of basic amino acids, several of which serve as processing points for the various peptide products derived from the common precursor (Figure 1). The precursor contains three regions of great sequence similarity (γMSH, αMSH, and βMSH) and may have evolved by gene triplication (Cohen et al., 1980). Nucleotide sequence analyses have also been reported for much of the human genomic DNA coding for pro-ACTH/endorphin (Chang, A. C. Y., et al., 1980) and for a portion of the mouse pituitary tumor cell pro-ACTH/endorphin mRNA (Roberts et al., 1979). The amino acid sequence of the NH_2-terminal region of a peptide corresponding to porcine 16K fragment (Hakanson et al., 1980) and the partial amino acid sequences of the NH_2-terminal regions of purified mouse 16K fragment (Keutmann et al., 1979) and biosynthetically labeled mouse and rat pro-ACTH/endorphin (Herbert et al., 1980; Gossard et al., 1980) have also been reported.

All of the peptide products derived from pro-ACTH/endorphin are secreted together in a coordinate fashion in vitro (Vale et al., 1978; Mains & Eipper, 1978; Przewlocki et al., 1979; Herbert et al., 1980; Eipper & Mains, 1980b; Krieger & Liotta, 1980), and evidence from in vivo studies is also consistent with coordinate secretion of all the peptide products derived from the common precursor (Tanaka et al., 1978; Hollt et al., 1979; Bertagna et al., 1980; Krieger et al., 1980; Mains & Eipper, 1980). In an attempt to define further what regions of the pro-ACTH/endorphin molecule may play an important role in physiology, we determined the amino acid sequences of two regions of mouse pro-ACTH/endorphin. Obtaining detailed structural information on mouse pro-ACTH/endorphin is especially important because many of the biosynthetic studies on the ACTH/endorphin common precursor molecule have been carried out with the mouse anterior pituitary tumor cell line AtT-20/D-16v (Eipper & Mains, 1980a; Herbert et al., 1980).

The NH_2-terminal region of mouse 16K fragment was found to be very similar to the homologous bovine and porcine molecules. In contrast to the close interspecies similarities in the 16K fragment, mouse γLPH was found to have a 22 amino acid deletion compared to bovine γLPH, and the pair of basic amino acids that mark the cleavage site to the NH_2-terminal side of βMSH in all known γ-lipotropins was found to be absent from mouse γLPH.

Materials and Methods

Incorporation of Labeled Amino Acids into Mouse Tumor Cell Molecules. AtT-20/D-16v cells were incubated in an enriched culture medium (XDMEM–CO_2; Mains & Eipper,

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[1] Abbreviations used: ACTH, adrenocorticotropin; βLPH, β-lipotropin; γLPH, γ-lipotropin; αMSH, α-melanotropin; βMSH, β-melanotropin; γMSH, γ-melanotropin; 16K fragment, the amino-terminal region of the common precursor to ACTH and βLPH, extending from the signal peptide to ACTH (Eipper & Mains, 1978; Nakanishi et al., 1979).

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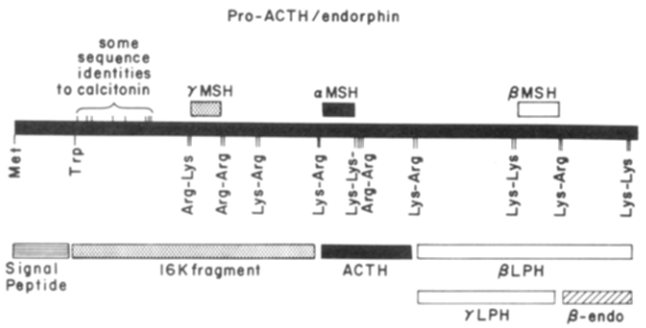

FIGURE 1: Diagram of the structure of pro-ACTH/endorphin. The structure of bovine pro-ACTH/endorphin is shown in the upper part of the diagram and is based on the nucleotide sequence of the cDNA as determined by Nakanishi et al. (1979); the locations of pairs of basic amino acids and the three melanotropin sequences are indicated. The major product peptides derived from pro-ACTH/endorphin in mouse anterior pituitary tumor cells are shown in the lower part of the diagram (Eipper & Mains, 1980a); the ACTH segment in mouse can occur with or without an oligosaccharide chain attached in its COOH-terminal region. The length of the signal peptide and thus the NH_2-terminal residue of the pro-ACTH/endorphin molecule were determined by comparing the partial amino acid sequences of mouse 16K fragment (Keutmann et al., 1979) and mouse pro-ACTH/endorphin (Herbert et al., 1980) and the corresponding bovine molecules (Cohen et al., 1980) to the complete amino acid sequence predicted for the bovine primary gene product (Nakanishi et al., 1979).

1979) with a single amino acid replaced by the corresponding radioactive amino acid at 30% of its normal concentration (Mains & Eipper, 1978). The following radioactive amino acids were obtained from New England Nuclear or Amersham: [^{3}H]alanine, [^{3}H]arginine, [^{14}C]arginine, [^{35}S]cystine, [^{3}H]glutamine, [^{3}H]glycine, [^{3}H]histidine, [^{3}H]leucine, [^{3}H]lysine, [^{3}H]proline, [^{3}H]serine, [^{3}H]threonine, [^{3}H]tyrosine, [^{3}H]tryptophan, and [^{3}H]valine. Cells were incubated for 16–30 h, depending on the label used, and the medium was removed from the cells and treated with protease inhibitors (phenylmethanesulfonyl fluoride and iodoacetamide; Mains & Eipper, 1978). Cells were further incubated in complete nonradioactive medium to allow the remainder of the radioactive peptides to be secreted (Mains & Eipper, 1978), and the medium was treated as described above. The pooled media were immunoprecipitated sequentially with antibodies to β-endorphin (affinity-purified Melinda), ACTH (affinity-purified Bertha and Roberta), and 16K fragment (antibody Georgie) (Eipper & Mains, 1978; Mains & Eipper, 1979). Immunoprecipitates were dissociated by boiling into 6 M guanidine hydrochloride and 5% 2-mercaptoethanol and fractionated by gel filtration on Sephadex G-75 (Pharmacia Fine Chemicals) in 10% formic acid and 0.1 mg/mL bovine serum albumin (Eipper & Mains, 1978). Aliquots were scintillation counted to locate the peaks of radioactive protein, and the pooled protein fractions were dried under reduced pressure at room temperature for sequence analysis.

Purification of Unlabeled Proteins. Mouse tumor cell βLPH, γLPH, and 16K fragment were purified from spent serum-free culture medium by ion-exchange chromatography on carboxymethylcellulose and gel filtration as described (Eipper & Mains, 1979; Keutmann et al., 1979). In some cases, ion-exchange chromatography was carried out on sulfopropyl-Sephadex (SP-C25-120; Pharmacia) instead of on carboxymethylcellulose. The sulfopropyl-Sephadex column was equilibrated with 0.01 M formic acid and 0.1% 2-mercaptoethanol, and the sample was loaded in the same buffer with 1.0% 2-mercaptoethanol. The column was washed with equilibration buffer and then with 0.01 M ammonium formate (pH 4.8). 16K fragment and γLPH were eluted together with a step to 0.10 M ammonium formate (pH 4.8), and βLPH and the various glycosylated forms of ACTH(1–39) were eluted during an exponential gradient to 0.10 M ammonium formate (pH 6.5). All buffers contained the stated molarity of formic acid (titrated to the desired pH with ammonium hydroxide) and 0.1% 2-mercaptoethanol. The major contaminant in the βLPH purified as described before (Eipper & Mains, 1979) is glycosylated ACTH(1–39); in order to remove more of the glycosylated ACTH(1–39), the pool of βLPH obtained from ion-exchange chromatography was fractionated by preparative-scale isoelectric focusing on Sephadex IEF (Pharmacia) (40 V/cm, 13 h) with a pH gradient of 6–8 with 5% Servalyt (Serva Feinbiochemica, Heidelberg, Federal Republic of Germany). Segments (0.7 cm) of gel were eluted in 0.1 M NH_4HCO_3, the single peak of immunoreactive βLPH (as detected by β-endorphin immunoassay) was pooled, and Servalyt was removed by gel filtration on Sephadex G-75 (superfine) in 0.1 M NH_4HCO_3.

Tryptic and Cyanogen Bromide Peptides. Tryptic peptides of γLPH were prepared and separated by gel filtration on Sephadex G-25 in 0.2 M NH_4HCO_3 as previously described (Eipper & Mains, 1979). Cyanogen bromide treatment was carried out with purified 16K fragment reduced and alkylated with iodo[^{3}H]acetic acid (Eipper & Mains, 1978; Keutmann et al., 1979), and the reaction products were analyzed by gel filtration on Sephadex G-75 in 6 M guanidine-HCl and 0.1 mg/mL bovine serum albumin (Eipper & Mains, 1978). Radioactive fractions were located by liquid scintillation counting. Compositional analyses of tryptic fragments and intact γLPH were carried out after acid hydrolysis in vacuo (6 N HCl, 110 °C, 24 h), by means of the Beckman Model 121MB automatic amino acid analyzer.

Sequencing Procedures. Automated Edman degradations (Edman & Begg, 1967) were carried out with the Beckman Model 890C sequencer with the 0.1 M Quadrol program (Beckman Instruments, Inc., Palo Alto, CA). Radiolabeled peptides (20 000–100 000 cpm) were dissolved in anhydrous trifluoroacetic acid, and 2–3 mg of myoglobin was added as carrier. The butyl chloride effluent containing the thiazolinone from each cycle was divided into aliquots for scintillation counting, and for conversion to the phenylthiohydantoin to determine repetitive yield from the myoglobin carrier. Unlabeled purified mouse βLPH or 16K fragment (40 nmol) was degraded by using the same program in the presence of 1–2 mg of Polybrene (Aldrich Chemicals). Phenylthiohydantoins were identified by gas chromatography (Pisano & Bronzert, 1969) and thin-layer chromatography on silica gel plates (Edman & Begg, 1967).

Results

Sequence of Mouse γLPH. Our previous studies on βLPH and γLPH purified from mouse pituitary tumor cell culture medium demonstrated that these molecules were approximately 20 amino acid residues shorter than known LPH molecules and suggested that the paired basic amino acids preceding the βMSH-like region were absent (Eipper & Mains, 1979). Initial Edman microsequencing experiments were aimed at defining the length and location of the apparent deletion in mouse γLPH and ascertaining the nature of the sequence changes around the processing site for βMSH. Most of the degradations were carried out with βLPH, which is longer than γLPH and less susceptible to extractive losses in later cycles of the sequencer runs. Roberts et al. (1979) determined the nucleotide sequence of a cDNA that coded for part of mouse tumor cell βMSH and most of β-endorphin; thus, it was known that mouse and bovine βMSH differed in

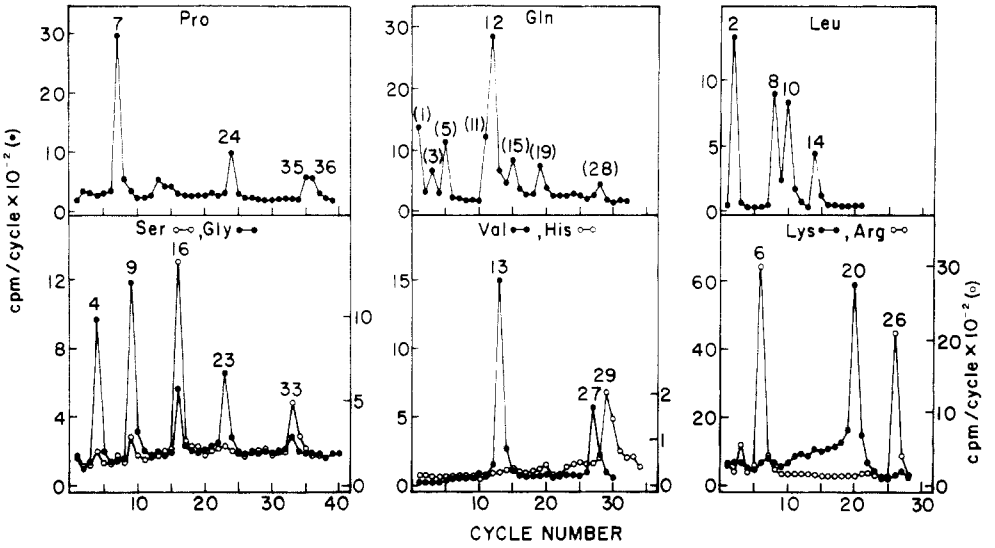

FIGURE 2: Edman degradations of immunoprecipitated mouse βLPH biosynthetically labeled with proline, serine, glycine, glutamine, valine, histidine, leucine, and lysine, and purified mouse βLPH labeled biosynthetically with arginine. Counts at serine positions in labeled glycine preparations are seen as a result of partial intracellular conversion of glycine to serine (see text). Peaks in the glutamine study numbered in parentheses represent glutamic acid positions, labeled as a result of intracellular conversion of radioactive glutamine to glutamic acid (see text).

amino acid sequence at several positions, but it was not possible to determine the length of mouse γLPH. The Edman degradations of biosyntheticaly labeled βLPH shown in Figure 2 provided the partial sequence $Gly^{23}Pro^{24}Val^{27}His^{29}Ser^{33}$-$Pro^{35,36}$. In the 93 amino acid bovine βLPH molecule, one finds the partial sequence $Gly^{45}Pro^{46}Met^{49}His^{51}Ser^{56}Pro^{57,58}$. On the basis of the work of Roberts et al. (1979), if mouse γLPH were the same length as bovine γLPH, one would expect to find the partial sequence $Gly^{45}Pro^{46}$-$Val^{49}His^{51}Ser^{55}Pro^{57,58}$. Thus, the βMSH-like region of mouse γLPH is displaced 22 residues closer to the NH_2 terminus than the βMSH-like region of bovine γLPH.

Automated Edman degradations of [^{3}H]Lys- and [^{14}C]-Arg-labeled mouse βLPH (Figure 2) placed arginine residues at positions 6, 26, and 31 (position 31 was identified in a separate Edman degradation of [^{3}H]arginine-labeled βLPH; not shown), and a single lysine at position 20. Thus, the paired basic residues preceding the βMSH region in the LPH molecules of other species were not found in mouse LPH; with the 22-residue deletion shown above, the paired basic residues would be expected to occur at positions 19 and 20. Instead, a single Lys residue occurs at position 20. The Arg residues at positions 26 and 31 correspond to $Arg^{48,53}$ in the βMSH region of bovine γLPH (Nakanishi et al., 1979), taking into account the 22 amino acid deletion in mouse γLPH.

The data indicating the absence of a pair of basic amino acids preceding the βMSH region in mouse γLPH are consistent with the results of compositional analyses (Eipper & Mains, 1979) and the pattern of tryptic peptides obtained from arginine- and lysine-labeled βLPH. As shown in Figure 3, one large, negatively charged tyrosine-containing peptide was seen in tryptic digests of [^{3}H]tyrosine-labeled mouse γLPH; in contrast, a single smaller, neutral tyrosine-containing tryptic peptide was obtained from [^{3}H]tyrosine-labeled rat γLPH. When tryptic digests of [^{3}H]Lys- and [^{3}H]Arg-labeled mouse βLPH were fractionated by gel filtration and paper electrophoresis, the distribution of radioactivity indicated the presence of one lysine and two arginine residues in the large, negatively charged tryptic peptide. The large, negatively charged tryptic peptide of LPH was purified by gel filtration (from a tryptic digest of unlabeled purified βLPH), and amino acid analysis indicated that it contained one lysine residue and two arginine residues. These observations are consistent with the identification of the large tryptic peptide (Figure 3) as mouse γLPH(1–26); this peptide includes trypsin-resistant basic residues at Arg^6 (adjacent to Pro^7) and Lys^{20} (flanked by Glu^{19} and $Asp^{21,22}$).

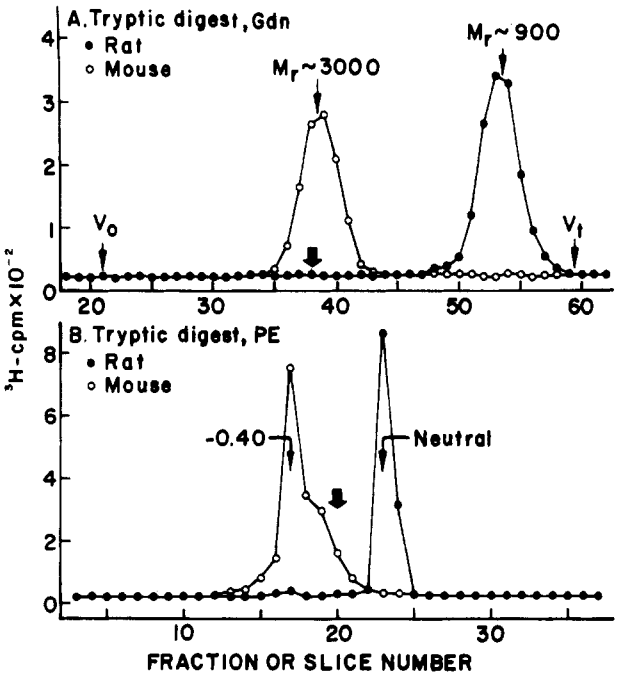

FIGURE 3: Analyses of tryptic digests of [^{3}H]tyrosine-labeled mouse and rat γ-lipotropins. Mouse pituitary tumor cells and rat intermediate pituitary cells were separately incubated in complete medium containing [^{3}H]tyrosine, and the respective γ-lipotropins were prepared by immunoprecipitation and gel filtration in 10%formic acid as described (Eipper & Mains, 1978; Mains & Eipper, 1979); for the tumor cells, β-endorphin-containing molecules were immunoprecipitated first, and the γLPH (which remained in the supernatant) was immunoprecipitated next. Radiolabeled molecules were digested with TPCK-trypsin. (A) Each digest was analyzed separately by gel filtration on Sephadex G-50 (superfine) in 6 M guanidine-HCl (Gdn), 0.2 mg/mL bovine serum albumin with blue dextran (V_0), glucagon (↓), and 2-mercaptoethanol (V_t) as internal markers (Eipper & Mains, 1978). (B) Each digest was analyzed separately by paper electrophoresis (PE) at pH 6.35; the heavy arrow marks the origin, and mobilities were calculated relative to lysine (1.00) and glycine (0).

Additional Edman degradations were carried out with βLPH labeled with leucine and glutamine (Figure 2), threo-

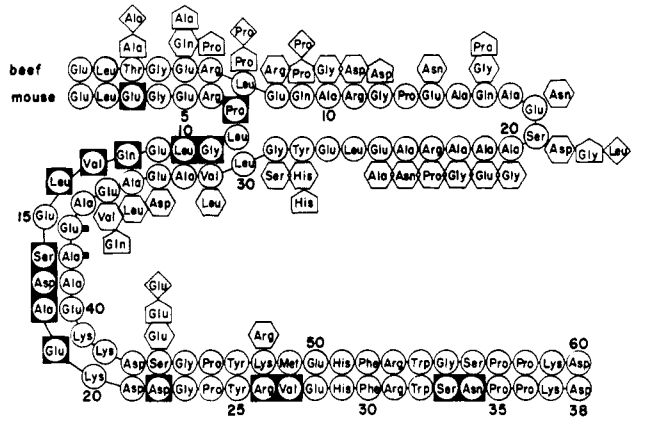

FIGURE 4: Comparison of mammalian γ-lipotropins. The sequences of bovine γLPH (open circles; Nakanishi et al., 1979) and mouse γLPH are compared. For positions where mouse γLPH is identical with bovine γLPH, the mouse residue is shown in open circles; for positions where mouse and bovine γLPH differ, the mouse residue is shown in an open circle in a dark square. For purposes of comparison, mouse γLPH has been depicted with a single 22-residue deletion corresponding to bovine γLPH(8–29) (see text). Where different from the corresponding bovine residue, the residues for human γLPH (hexagon; Li & Chung, 1976), porcine γLPH (pentagon; Graf et al., 1971), and whale γLPH (diamond; Kawauchi et al., 1980) are indicated. The amino acid sequence predicted for human γLPH from the genomic DNA coding for this protein (Chang, A. C. Y., et al., 1980) shows substantial differences from the amino acid sequence reported by Li & Chung (1976). Sheep and camel γLPH are reported to be identical with bovine γLPH (Li et al., 1977; Bunney, 1979). The black tab markers next to residues 37 and 38 of bovine γLPH indicate that these two residues were not present in the amino acid sequence determined for purified bovine γLPH but were present in the amino acid sequence predicted from the nucleotide sequence (Nakanishi et al., 1979).

nine, and tryptophan (not shown). Some intracellular conversion of glutamine to glutamic acid and glycine to serine was observed during the lengthy incubations used. On the basis of analyses of Pronase digests of a water extract of cells labeled with [^{3}H]glutamine, 27% of the radioactivity incorporated into protein had been converted into [^{3}H]glutamic acid; no detectable conversion (<1%) of [^{3}H]glutamine to [^{3}H]glutamic acid occurred in the culture medium during the incubations. Similarly, about 25% of the radioactivity derived from [^{3}H]glycine had been converted into [^{3}H]serine.

The remaining positions in mouse γLPH not assigned by the radioactive microsequencing procedure were identified in a series of 25–30 cycle degradations with three separate preparations of 40 nmol of purified mouse βLPH. Most of the phenylthiohydantoins were identified by thin-layer chromatography, including Glu, Asp, and Ala. Glutamic acid positions coincided with those indicated by the degradation of [^{3}H]Gln-labeled βLPH (Figure 2), although recoveries were lower at cycle 3 than at other cycles in both labeled and unlabeled preparations. A repeat degradation of [^{3}H]Gln-labeled βLPH showed a full complement of Glu at cycle 3, permitting the assignment of Glu at that position.

The sequence of mouse γLPH is compared with the sequence of γLPH from other mammalian species in Figure 4. The sequence comparison has been depicted with the deletion in the mouse γLPH molecule corresponding to bovine γLPH(8–29). The initial portion of mouse γLPH (residues 1–7) and the βMSH-like region of mouse γLPH (residues 21–38) clearly correspond to known γLPH sequences, but exact placement of the deletion and the assumption that it occurs as one stretch of 22 amino acid residues are arbitrary.

As shown in Figure 4, mouse γLPH is 38 residues long (M_r 4439), and thus mouse βLPH has 71 residues (M_r 8144).

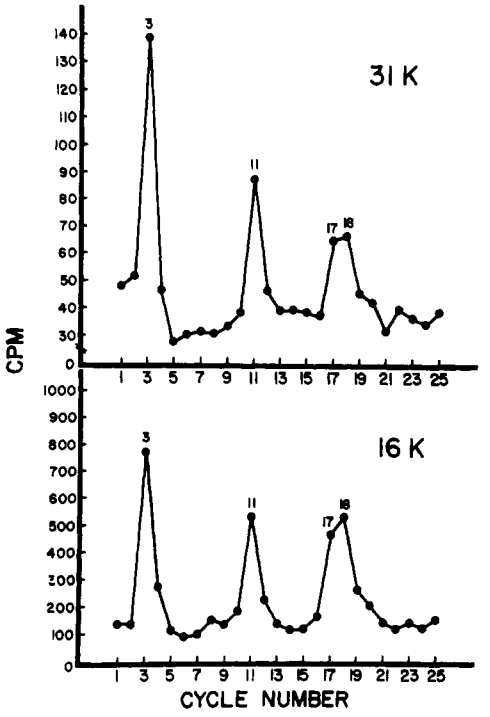

FIGURE 5: Edman degradations of immunoprecipitated mouse pro-ACTH/endorphin (31K) and 16K fragment biosynthetically labeled with leucine.

These results are in excellent agreement with the previous estimates of 39 residues (M_r 4600 ± 200) for mouse γLPH and 72 residues (M_r 8200 ± 250) for mouse βLPH, based on gel filtration in 6 M guanidine-HCl (Eipper & Mains, 1979). The amino acid composition of mouse γLPH shown in Figure 4 also agrees closely with the previous amino acid composition data for purified mouse γLPH and βLPH (Eipper & Mains, 1979).

NH_2-Terminal Sequence of Mouse 16K Fragment and Pro-ACTH/Endorphin. As discussed above, 16K fragment is one of the final products of the posttranslational processing of pro-ACTH/endorphin in the anterior pituitary. In addition to carrying out sequence studies on 16K fragment, we also performed limited sequencing studies on pro-ACTH/endorphin and the ACTH biosynthetic intermediate in order to determine the location of the 16K fragment within the larger precursor forms [ACTH biosynthetic intermediate is the molecule created when βLPH is cleaved from pro-ACTH/endorphin (Figure 1) (Eipper & Mains, 1980a)]. Figure 5 shows separate degradations of [^{3}H]leucine-labeled 16K fragment and pro-ACTH/endorphin (31K); similar profiles of radioactivity ($Leu^{3,11,17,18}$) were found for the two molecules, confirming the NH_2-terminal location of 16K fragment within pro-ACTH/endorphin.

Most of the NH_2-terminal residues of the 16K fragment were assigned by degradations of separate preparations of 40 nmol of unlabeled 16K fragment. Although these purified preparations had shown evidence of charge heterogeneity by isoelectric focusing (not shown), a single uniform sequence was obtained upon Edman degradation. The charge heterogeneity in the isolated 16K fragment samples may be due to microheterogeneity in the oligosaccharide chain, or to differences at the COOH-terminal end of the peptide backbone.

Additional positions not readily assigned from the unlabeled 16K fragment degradations were determined by analysis of separate preparations of immunoprecipitated 16K fragment biosynthetically labeled with cystine, serine, threonine, tryptophan, and valine (Figure 6), and arginine, glutamine, lysine,

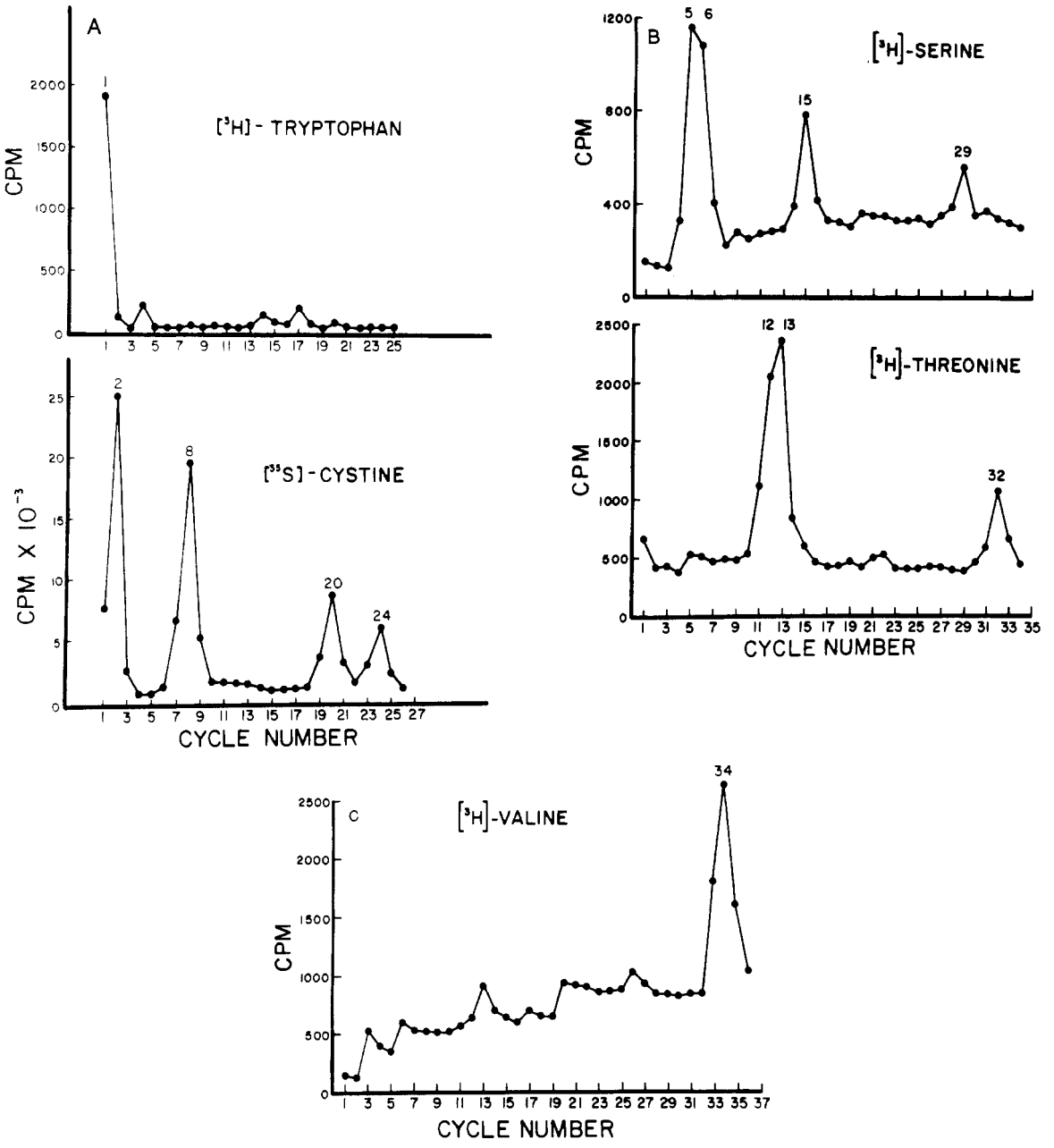

FIGURE 6: Edman degradations of purified mouse 16K fragment biosynthetically labeled with tryptophan and immunoprecipitated mouse 16K fragment biosynthetically labeled with cystine, serine, threonine, and valine.

and proline (not shown). The location of half-cystine residues at positions 2, 8, 20, and 24 was determined previously by degradation of purified 16K fragment labeled by reaction with iodo[^{3}H]acetic acid (Keutmann et al., 1979).

These degradations provided sequence assignments for 27 of the first 29 residues, as well as Thr^{32} and Val^{34} (Figure 7). Among the 29 residues assigned for mouse 16K fragment, only one position differed from the sequence of the corresponding bovine molecule predicted from the nucleotide sequence (Leu^{26} in mouse replaced bovine Pro^{26}), and this substitution could represent a single base change in the codon (CUC in mouse for CCC in bovine).

Through the first 29 residues of mouse 16K fragment, only two positions were not assigned (Asn^{16} and Asp^{27} in the bovine molecule; Figure 7). Although it was not possible to document Asp^{27} because of the difficulty of biosynthetic labeling with Asp and Asn (Eipper & Mains, 1977), further data indicate that position 27 in mouse 16K fragment is not methionine as reported by Herbert et al. (1980). On the basis of peptide analyses of [^{3}H]methionine-labeled 16K fragment (Eipper & Mains, 1978) and on amino acid analyses of purified 16K fragment (not shown), there is one methionine per mole of mouse 16K fragment. Cyanogen bromide cleavage of purified mouse 16K fragment labeled at Cys^{2,8,20,24} by reaction with iodo[^{3}H]acetic acid generated a labeled peptide with a mass of approximately 6400 daltons (Figure 8). On the basis of the nucleotide sequence of Nakanishi et al. (1979), cyanogen bromide cleavage of the bovine protein corresponding to mouse 16K fragment would generate a 53-residue (M_r 5902) peptide. Thus, the single methionine residue in mouse 16K fragment appears to occur in a position within the γMSH region, closely analogous to the location of the single methionine residue in the corresponding bovine molecule (Figure 1).

Discussion

The growing fund of structural information on pro-ACTH/endorphin is making possible more meaningful sequence comparisons and inferences regarding potential functions for different regions of the molecule. The two portions examined in this investigation, γLPH and the NH_2-terminal

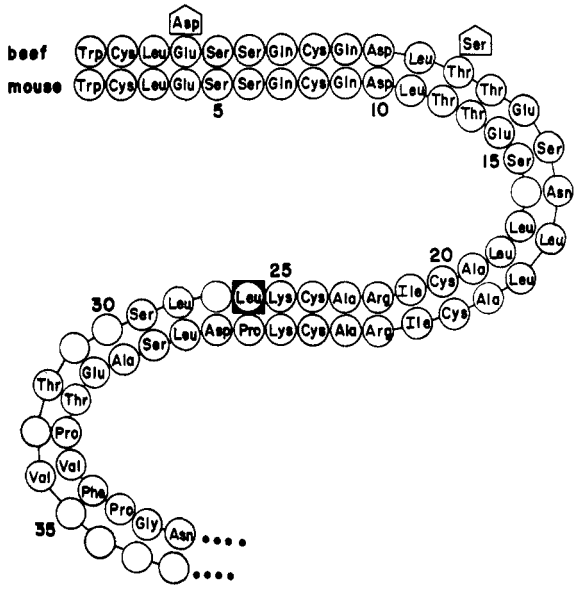

FIGURE 7: Comparison of mammalian peptides corresponding to mouse 16K fragment. The amino acid sequence predicted for the NH_2-terminal region of bovine pro-ACTH/endorphin (Nakanishi et al., 1979) has been renumbered to take into account the existence of a signal sequence. Differences between the mouse and bovine sequences are indicated as described in Figure 4. The NH_2-terminal 35 residues of a porcine peptide corresponding to mouse 16K fragment were sequenced by Hakanson et al. (1980); where different from the corresponding bovine residue, the residues for the porcine molecule are indicated in pentagons.

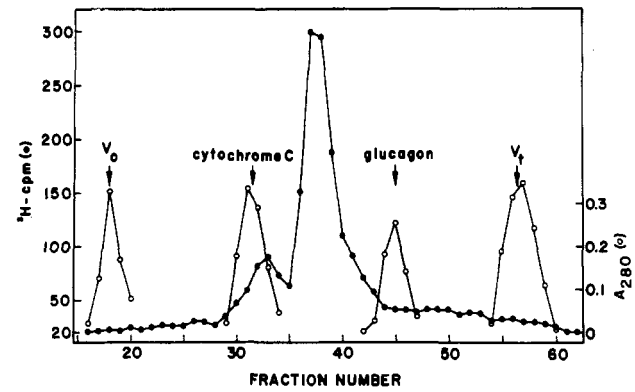

FIGURE 8: Cyanogen bromide fragments of purified mouse 16K fragment labeled at cysteine residues (positions 2, 8, 20, 24) by reaction with iodo[^{3}H]acetic acid. After treatment with cyanogen bromide (see Materials and Methods), the reaction mixture was lyophilized and analyzed by gel filtration on Sephadex G-75 in 6 M guanidine-HCl and 0.2 mg/mL bovine serum albumin. Blue dextran (V_0), cytochrome *c*, glucagon, and 2-mercaptoethanol (V_t) were included as internal standards. The label eluted predominantly in a single peak of approximately 6400 daltons (fractions 36–40), consistent with location of the single methionine in mouse 16K fragment at a position closely analogous to that in the bovine molecule (see text). The small peak of radioactivity in fractions 30–34 represents intact 16K fragment.

region of 16K fragment, represent a particular contrast in sequence conservation which could reflect their relative physiological roles or importance.

Previous amino acid sequence data for mouse γLPH were limited to the COOH-terminal 15 residues and were derived from the nucleotide sequence of a segment of cloned cDNA obtained by Roberts et al. (1979). Besides the completion of the amino acid sequence of γLPH, the work described here confirms that the major cell product produced by AtT-20 cells is the same as the protein coded for by this single cloned cDNA.

Our finding of a 22-residue deletion within mouse γLPH confirms earlier gel filtration and peptide mapping data (Eipper & Mains, 1979) showing that mouse γLPH and βLPH were shorter than the respective hormones from pig, beef, whale, camel, and human pituitary (Kawauchi et al., 1980; Graf et al., 1971; Li & Chung, 1976; Li et al., 1977; Nakanishi et al., 1979; Chretien et al., 1979). The fact that six of the first seven residues of mouse γLPH are identical with residues in known mammalian γ-lipotropins indicates that proteolysis of the NH_2 terminus of γLPH is not responsible for the shortening of mouse γLPH. The nucleotide sequence of the human genomic DNA coding for pro-ACTH/endorphin (Chang, A. C. Y., et al., 1980) indicates that, compared to bovine γLPH, human γLPH has a 6-residue deletion and a 1-residue insertion in the same region where the deletion occurs in mouse γLPH. In one form of human growth hormone, a deletion of 15 residues appears to arise through variation in splicing at an intron–exon junction during processing of the genomic DNA (Lewis et al., 1980; Fiddes et al., 1979). This appears not to explain the deletion in mouse γLPH, as there is no intron–exon junction in this region of human or bovine pro-ACTH/endorphin genomic DNA (Chang, A. C. Y., et al., 1980; Nakanishi et al., 1980).

Previous work had indicated that mouse and rat pituitary did not contain a conventional βMSH (Shapiro et al., 1972; Lowry & Scott, 1975; Jackson & Lowry, 1980; Eipper & Mains, 1979). The βMSH region of mouse γLPH (positions 21–38) is clearly homologous to that of bovine βMSH; the two species differ at 5 of 18 positions (positions 2, 6, 7, 13, and 14 of βMSH). On the basis of the cDNA analyses of Roberts et al. (1979), the substitutions at positions 7, 13, and 14 of βMSH occurred by single base changes. Although bovine βMSH has Ser at position 2, porcine, human, and whale βMSH have Glu at position 2, and the Asp found at the corresponding position in the βMSH region of mouse γLPH represents a conservative substitution. Similarly, while bovine βMSH has a Lys at position 6, mouse, human, monkey and turkey βMSH have Arg at this position (Li & Chung, 1976; Chang, A. C. Y., et al., 1980; Chang, W. C., et al., 1980).

The paired basic amino acids that mark the proteolytic cleavage site at the NH_2-terminal end of βMSH in other LPH molecules are absent from mouse LPH (Figures 2, 3, and 4). On the basis of the structures of precursors to other peptide hormones such as insulin, parathyroid hormone, calcitonin, glucagon, gastrin, and somatostatin, paired basic amino acids occur at proteolytic cleavage sites (Steiner et al., 1980; Potts et al., 1980; Goodman et al., 1980; Amara et al., 1980; Tager et al., 1980; Agarwal & Noyes, 1980). A mutant form of human proalbumin that lacks the paired basic amino acids separating the propeptide from albumin does not undergo proteolytic processing to albumin (Brennan & Carrell, 1978). Thus, lack of paired basic residues to the NH_2-terminal side of βMSH in mouse γLPH might be expected to preclude proteolytic cleavage at this site in tissues, such as the intermediate pituitary, that normally produce βMSH in other species. Under basal conditions, the AtT-20 tumor cells (which are derived from the anterior pituitary) cleave less than 3% of the γLPH to smaller products (Mains & Eipper, 1981).

Rat γLPH appears to be similar in size to mouse γLPH (Jackson & Lowry, 1980; Przewlocki et al., 1979; Eipper & Mains, 1979). However, rat γLPH is immunologically distinct from mouse γLPH, and trypsin treatment of [^{3}H]tyrosine-labeled rat and mouse γLPH produces different peptide patterns (Figure 3). Partial sequence data for rat γLPH (Seidah et al., 1978, 1980) suggest that it differs from mouse γLPH at two of the six positions where comparisons can be made.

The high degree of species specificity in the NH_2-terminal region of γLPH, and the variable length of γLPH from dif-

ferent species, suggests that this region of the pro-ACTH/endorphin molecule may not serve a physiologically important function as a circulating hormone.

16K fragment is one of the final products of posttranslational processing of pro-ACTH/endorphin in the anterior pituitary. Since Edman degradations of [^{3}H]leucine-labeled 16K fragment and pro-ACTH/endorphin gave similar patterns (Figure 5), no substantial further proteolytic processing occurs at the NH_2 terminus of tumor cell pro-ACTH/endorphin during the production of 16K fragment. Our data and the data of Herbert et al. (1980) for mouse tumor cell pro-ACTH/endorphin immunoprecipitated from cell extracts are consistent with removal of a 26-residue signal peptide by cleavage at Gly^{-1} Trp^{+1} of the ribosomal precursor sequenced by Nakanishi et al. (1979). Substantial amounts of a glycopeptide corresponding to the NH_2-terminal region of pig pro-ACTH/endorphin were also found to have Trp at position 1 (Hakanson et al., 1980). In contrast, Gossard et al. (1980) reported sequence heterogeneity at position 1 of rat pro-ACTH/endorphin; both Trp and Arg were found. Gossard et al. (1980) incubated rat intermediate-posterior pituitarties with labeled amino acids and subjected the whole tissue extract to Edman degradation; no purification of the ACTH/endorphin precursor was carried out. It was not possible to document any arginine at position 1 in the mouse tumor cell molecules studied in this work.

In our studies of mouse tumor cell 16K fragment and pro-ACTH/endorphin [Figures 7 and 8; also see Keutmann et al. (1979)], about 20% of the secreted tumor cell molecules lacked Trp^1 and had half-cystine at their NH_2 terminus. When tumor cells were incubated with labeled amino acids for longer times, Trp^1 was absent from as much as 40–50% of the 16K fragment. Rapid removal of Trp^1 can be effected by mild treatment of 16K fragment with aminopeptidase M (unpublished experiments). In rat pituitary cell extracts, Gianoulakis et al. (1979) reported several products related to the NH_2-terminal region of pro-ACTH/endorphin that lacked NH_2-terminal tryptophan.

In contrast to the extensive differences between species in the γLPH region, these data show marked conservation between the mouse 16K fragment and the other species for which nearly complete 16K fragment sequence data are available. In the first 29 positions of 16K fragment, the mouse and bovine sequences were documented to differ at only one position (26); the mouse and porcine sequences differed at only three positions (4, 12, and 26). Each of these amino acid sequence changes could occur by single nucleotide changes from the bovine codons. The substitution of Leu^{26} in mouse 16K fragment for Pro^{26} in the beef and pig molecules would account for the pattern of iodo[^{3}H]acetic acid labeled peptides obtained previously by tryptic digestion of mouse 16K fragment (Keutmann et al., 1979).

Since most sequence data encompass only the NH_2-terminal third of the 16K fragment, it cannot be predicted whether close sequence homologies extend through the remaining portions of 16K fragment. There is evidence from studies of tryptic and cyanogen bromide peptides favoring the existence of a γMSH region in mouse 16K fragment closely resembling bovine γMSH in structure and in placement within the 16K fragment. The nucleotide sequences for bovine and human pro-ACTH/endorphin show identical structures and positioning of γMSH within 16K fragment (Nakanishi et al., 1979; Chang, A. C. Y., et al., 1980). In disagreement with these data, Herbert et al. (1980) reported that the single methionine residue in mouse 16K fragment was at position 27; the occurrence of Met at position 27 would require a complete change in the codon from that found in bovine cDNA (GAC in beef to AUG), and would preclude the existence of a region identical with γMSH in the mouse molecule. Gianoulakis et al. (1979) and Seidah et al. (1980) reported that there were no methionine residues in rat 16K fragment, again indicating the absence of a segment identical with γMSH. In contrast, our studies demonstrated the presence of a single methionine residue in rat 16K fragment in a tryptic peptide closely resembling that expected from bovine γMSH (Mains & Eipper, 1979; unpublished experiments).

The partial amino acid sequence data of Herbert et al. (1980) indicated that position 7 of mouse 16K fragment was Val (codon GUX); the bovine molecule has Gln^7 (codon CAG; Nakanishi et al., 1979). Our analyses of both unlabeled and [^{3}H]Gln-labeled mouse 16K fragment identified position 7 as Gln, as in bovine 16K fragment. In our analyses of [^{3}H]valine-labeled 16K fragment, no radioactivity was released at cycle 7, while in the same degradation, radioactivity was released at cycle 34 (Figure 6); bovine 16K fragment has Val^{34}. The partial sequence data reported by Seidah et al. (1980) for rat 16K fragment show Leu^{26} (as in the mouse) and Arg^{25} (Lys^{25} in beef, pig, and mouse). Seidah et al. (1980) also reported Phe^4 and Tyr^{14} (replacing $Glu^{4,14}$ in beef and mouse); the occurrence of Phe^4 and Tyr^{14} in rat 16K fragment would represent changes of three and two nucleotides, respectively, from the codons found in the bovine cDNA (Nakanishi et al., 1979).

Studies of potential physiological actions of 16K fragment have largely concentrated on peptides from the γMSH region in the mid portion of the molecule, and these peptides have been shown to have distinct effects on adrenal steroidogenesis (Pedersen et al., 1980). When potential biological actions for the highly conserved NH_2-terminal region of 16K fragment are considered, a clue may be offered by the similarities in the amino acid sequence of 16K fragment and calcitonin. The cysteines spaced seven residues apart near the NH_2 terminus (positions 2 and 8) and the sequence -Glu-Thr-Pro- at residues 31–33 of bovine 16K fragment align precisely with the NH_2 and COOH termini of the 32-residue bovine calcitonin molecule (Keutmann et al., 1980) (Figure 1).

We have recently prepared several synthetic fragments representing the calcitonin-like NH_2-terminal portion of bovine 16K fragment (Keutmann et al., 1980). These synthetic peptides, as well as purified mouse 16K fragment, will enable broad screening to be undertaken, both in vivo and in vitro, for as yet undetected hormonal activities within this region of the pro-ACTH/endorphin molecule.

Added in Proof

Since submission of this manuscript, the nucleotide sequence of a cloned genomic rat DNA coding for pro-ACTH/endorphin has been reported by Drouin & Goodman (1980). On the basis of these results, rat γLPH is also 38 residues long and lacks paired basic amino acids preceding its βMSH-like segment. The rat and mouse γLPH molecules would be expected to differ at 6 of the 38 positions; at position 21, replacement of Asp (mouse) by Ala (rat) would explain the vastly different tyrosine-containing tryptic peptides obtained from rat and mouse γLPH (Figure 3). Five of the six sequence differences between mouse and rat γLPH could have occurred by single base changes in the respective codons. The Drouin & Goodman (1980) data further indicate that rat 16K fragment has a single methionine in a region identical with that of bovine γMSH and a valine at position 34, in full agreement with the data for mouse 16K fragment in this paper. Of the 12 positions where the mouse and predicted rat 16K fragment

sequences can be compared, 11 are identical, and one could represent a single base change in the codon (position 25, where mouse and bovine are Lys but rat is Arg).

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