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Growth hormones. II. Structure—function relationships

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Summary — Localization of the somatotropic activity of growth hormones from several species and from different organs was attempted using different approaches. Sequences were compared in order to detect one or several regions with a common homology. The technique of peptide recombinants as well as chemical changes affecting some amino acids was applied to these hormones; the biological function *in vivo* of growth or binding to somatotropic receptors was then estimated. The few data available on biosynthetic molecules and secondary structures of natural growth hormones are reported. This study indicates the somatotropic function of particular sites.

growth hormones — somatotropic activity — localization

Résumé — **Hormones de croissance. II. Relations structure—fonction.** *L'étude des relations structure-fonction d'hormones à activité de croissance, d'origines hypophysaires (GH) ou placentaires (CS ou PL) et d'espèces variées, nous a conduit à formuler quelques hypothèses. Une séquence particulière et restreinte est-elle suffisante pour révéler une activité hormonale ? Existe-t-il un ou plusieurs sites biologiquement actifs impliqués dans la liaison hormone—récepteur ? Quelle est l'incidence de la conformation moléculaire sur la fonction somatotrope ? Pour tenter de répondre à ces questions, nous avons proposé les approches suivantes :*

— *l'étude comparée des séquences d'hormones de croissance et de variants ainsi que des données, encore trop restreintes, sur la structure tridimensionnelle de quelques hormones nous a permis de mettre en évidence, d'une part, des régions d'homologie commune et de dégager, d'autre part, des déterminants structuraux caractéristiques de domaines à vocation somatotrope;*

— *l'activité biologique d'hormones de croissance soumises à l'action d'enzymes protéolytiques ou dont certains résidus d'acides aminés ont été altérés par des modifications chimiques, a été estimée soit in vivo, soit par liaison à des récepteurs somatotropes;*

— *la méthode originale utilisant les recombinants peptidiques entre hormones pourvues ou non d'activité de croissance a été appréciée pour la localisation de sites actifs, neutres, voire même inhibiteurs dans l'activité de croissance;*

— *l'emploi de peptides synthétiques, d'anticorps monoclonaux et de molécules biosynthétiques a complété cette étude.*

hormones de croissance — activité somatotrope — localisation

Introduction

Study of structure—function relationships must be carried out on physically and chemically homogeneous as well as on biologically active products. Increasing sophistication in purification methods, such as HPLC (high performance liquid chromatography), allows the highly purified polypeptide hormones required for such studies to be obtained. Elucidation of the primary structure of proteins has become easier with the availability of the micro-sequencing devices which require only very small amounts of product, on the nanomole scale. During the last few years, prediction of secondary and tertiary structures has improved, and allows assessment of the spatial conformation of polypeptides. The criteria used to define biological activities are based on the well known multiple action of GHs (see "Growth Hormones. I. Polymorphism"; Charrier and Martal, 1988, Tables II and III).

Growth-promoting activities were demonstrated *in vivo* in hypophysectomized rats by the tibia test (Greenspan *et al.*, 1949) or the weight gain test (Wilhelmi, 1973), and *in vitro* through hormone affinity to hepatocyte cellular membranes (Tsushima and Friesen, 1973). Insulin activity was demonstrated by the binding of hormones to adipocyte cellular membranes (Posner *et al.*, 1974; Fagin *et al.*, 1980) or lymphocytes (Lesniak *et al.*, 1973). Such binding is used in the so-called radioreceptor assay (RRA) to demonstrate and quantify biological activities and is easier than the conventional and time consuming *in vivo* methods. Somatotropic effect also refers to somatomedin or insulin-like growth factor (IGFs) stimulation. Human placental lactogen and growth hormone increase IGF-I on human fetal pancreas in tissue culture (Swenne *et al.*, 1987).

The use of monoclonal antibodies specific for an epitope leads to a better knowledge of immunological determinants. Analysis of immunological activity sites will not be studied here. However, inhibition of biological activities may be obtained using monoclonal antibodies. Synthesis of polypeptide hormones similar to natural hormones is now possible as a result of advances in biotechnological methods.

The purpose of the present paper is to study structure—function relationships of growth hormones of pituitary (GHs) and placental origin (placental lactogen [PL] or chorionic somatomammotropin [CS] and human placental growth hormone [hPGH]) from several species. Some of these hormones possess another biological activity: for example, hGH also has a lactogenic activity. Some placental hormones have either only a lactogenic activity, such as human (hPL), rat (rPL) and mouse (mPL) placental lactogen (Friesen, 1965; Robertson and Friesen, 1975; Talamantes, 1975), or both growth and lactogenic activities, such as bovine (Forsyth, 1973; Bolander and Fellows, 1976; Kelly *et al.*, 1976), caprine (Becka *et al.*, 1977; Currie *et al.*, 1977), ovine (Handwerger *et al.*, 1974; Chan *et al.*, 1976; Martal and Djiane, 1976; Martal, 1978), simian (Shome and Friesen, 1971) and guinea-pig (Talamantes, 1975, Kelly *et al.*, 1976) chorionic somatomammotropins (CS).

The study of hormone structure—function relationships gives rise to many questions. The question may be raised as to whether there exists one or several biologically active sites, as already shown in some enzyme proteins. The following preliminary hypotheses have been put forward on structure—function relationships.

Are there particular regions of a hormone liable for specific biological activity and what is the function of the hormonal

molecule? In order to answer these questions, we made a comparative analysis of already known GH primary structures. Some recently obtained information on molecular conformation of some GHs may also constitute an original approach to the understanding of structure—function relationships. Proteolytic enzymes are utilized in the so-called peptide recombinant method, which allows localization of active sites of growth hormones in the same way as utilisation of polypeptide analogues. Do the chemical modifications altering some amino acid residues affect the biological activity of these hormones? Does mutagenesis modify biological hormonal activity?

Analysis of invariants

Growth hormones

Pituitary growth hormones are globular holoproteins consisting of about 191 amino acids with a predominantly α -helix structure ($\approx 50\%$). They contain a single tryptophan residue at position 86 and 4 cysteine residues which form a small loop in the COOH-terminal part (Cys 182—Cys 189) and a large one in the NH₂-terminal part (Cys 53—Cys 165) (Table I). In all the already identified growth hormones, the NH₂-terminal end mainly consists of phenylalanine or sometimes alanine residues.

To our knowledge, growth hormone sequences have already been determined in several species: horse (Zakin *et al.*, 1973; Daurat-Larroque *et al.*, 1977), man (Bewley *et al.*, 1972), sheep (Li *et al.*, 1972), pig (Seeburg *et al.*, 1983), beef (Wallis, 1973; Graf and Li, 1974a), rat (Wallis and Davis, 1976; Barta *et al.*, 1981), chicken (Souza *et al.*, 1984), mouse (Linzer and Talamantes, 1985),

monkey (Li *et al.*, 1986), sei whale (Pankov *et al.*, 1982) and fin whale (Tsubokawa and Kawauchi, 1985). The method of comparison of 8 GH sequences consisted of lining up amino acids while complying with the largest position identity between one another according to Dayhoff (1976): human growth hormone (hGH), monkey growth hormone (MGH), ovine growth hormone (oGH), bovine growth hormone (bGH), equine growth hormone (eGH), chicken growth hormone (cGH), rat growth hormone (rGH) and mouse growth hormone (mGH). In this text, amino acid numbering of GH was based on primary sequence of hGH (Table I).

Some characteristics can be deduced from this comparison. There exists a large sequence similarity between human and monkey GHs (Li *et al.*, 1986), since only 4 amino acid residues differ at positions 105, 107, 133 and 173 due to the mutation of only one codon nucleotide.

hGH exhibits a 25% difference in the residue composition as compared to bGH (Bewley and Li, 1970; Wallis, 1975). In contrast, ovine and bovine growth hormones are quite similar since only 1% of their residues are different, indicating a close relationship between these 2 species (Wallis, 1975). A 77% similarity was observed by Souza *et al.* (1984) between chicken and bovine GH (Wallis, 1973). Analysis of rGH and mGH sequences shows a 95% and 92% identity, respectively, with that of ruminants. The rat and mouse GH sequences only differ from one another in 2 amino acids at positions 11 and 186. Only avian GH possesses an isoleucine residue as COOH-terminal amino acid, while phenylalanine is common to all the considered mammalian GHs. It has been suggested that PRLs and GHs are derived from a common initial peptide consisting of 25—50 amino acids, which by successive duplications would have generated these hormones

Table I. Comparison of primary sequences of different growth hormones.

	10	20	30	40	50
hGH	H-Phe	Thr	Ile	Pro	Leu
MGH	H-Phe	Pro	Ile	Pro	Leu
cGH	H-Phe	Ala	Met	Ser	Leu
bcGH	H-Phe	Pro	Ala	Ser	Leu
ecGH	H-Phe	Ala	Met	Ser	Leu
rcGH	H-Phe	Ala	Met	Ser	Leu
mGH	H-Phe	Pro	Ala	Ser	Leu
hGH	Asp	Thr	Gln	Glu	Leu
MGH	Asp	Thr	Gln	Glu	Leu
cGH	Asp	Thr	Lys	Glu	Leu
bcGH	Asp	Thr	Lys	Glu	Leu
ecGH	Glu	Thr	Lys	Glu	Leu
rcGH	Asp	Thr	Lys	Glu	Leu
mGH	Asp	Thr	Lys	Glu	Leu
hGH	Ser	Leu	Cys	Leu	Lys
MGH	Ser	Leu	Cys	Leu	Lys
bcGH	Ala	Phe	Cys	Leu	Lys
ecGH	Ala	Phe	Cys	Leu	Lys
rcGH	Ala	Phe	Cys	Leu	Lys
mGH	Ala	Phe	Cys	Leu	Lys
hGH	Leu	Arg	Ile	Gln	Val
MGH	Leu	Arg	Ile	Gln	Val
bcGH	Leu	Arg	Ile	Gln	Val
ecGH	Leu	Arg	Ile	Gln	Val
cGH	Leu	Arg	Ile	Gln	Val
rcGH	Leu	Arg	Ile	Gln	Val
mGH	Leu	Arg	Ile	Gln	Val
hGH	Leu	Arg	Ile	Gln	Val
MGH	Leu	Arg	Ile	Gln	Val
bcGH	Leu	Arg	Ile	Gln	Val
ecGH	Leu	Arg	Ile	Gln	Val
cGH	Leu	Arg	Ile	Gln	Val
rcGH	Leu	Arg	Ile	Gln	Val
mGH	Leu	Arg	Ile	Gln	Val
hGH	Leu	Arg	Ile	Gln	Val
MGH	Leu	Arg	Ile	Gln	Val
bcGH	Leu	Arg	Ile	Gln	Val
ecGH	Leu	Arg	Ile	Gln	Val
cGH	Leu	Arg	Ile	Gln	Val
rcGH	Leu	Arg	Ile	Gln	Val
mGH	Leu	Arg	Ile	Gln	Val

(Niall *et al.*, 1971). Repetition of a model with a very close amino acid composition is found in hGH at positions 15—32, 93—110, 128—147, 163—180 (Table II and Table III). These observations regarding the existence of 4 homologous internal regions suggest that they might be involved in hormonal biological activities.

These regions do not always correspond to particularly well conserved segments of GHs and PRLs. Thus, examination of 7 mammalian prolactin sequences and 9 growth hormones (of which 8 are from mammals) and analysis of either homologous or closely related invariant amino acids led to identification by Nicoll *et al.* (1986) of several high identity clusters separating poorly homologous regions. These clusters are located in fragments 8—24, 51—68, 69—86, 114—125 and 158—167. Only 2 of them partly correspond to 2 regions identified by Niall, including residues 15—32, 163—180 (Table II).

Recently, Kawauchi and Yasuda (1987) have compared amino acid sequences of fish, chicken and mammalian GHs and PRLs, according to 204 alignment positions. They have delineated 4 highly conserved domains for GHs in alignment positions: A (5—37), B (49—89), C (104—126) and D (138—181) (Tables II and III).

Three of these domains, A, B and C, partially overlap those of Nicoll, and only A and D imperfectly correspond to the patterns identified by Niall (15—32 and 163—180). It would be interesting to determine whether some of these highly conserved domains possess a specific somatotrophic-like bioactivity.

Table II gives a consensus sequence of the GH family (Con GH). If we take into account functional homologies according to Dayhoff (1976) between some amino acids such as Arg-Lys, Arg-Glu, Leu-Ile-

Val, Ser-Thr, and amino acids with frequent or very frequent mutation such as Asp-Asn, Met-Val, Asn-Ser, His-Asn, Val-Ile, Gln-Arg, His-Gln, Ile-Phe, Met-Arg, we obtain a molecular archetype with a 70% common primary structure (Martal, 1980).

The 20K variant of hGH

This variant was first noted by Lewis *et al.* (1978). These authors emphasized the deletion of amino acids 32—46 (Lewis *et al.*, 1980). The features of this variant were reviewed by Charrier and Martal (1988). It exerts *in vivo* somatogenic effects analogous to the 22K form of hGH, as shown by the rat tibia test (Spencer *et al.*, 1981) and the weight gain test (Lewis *et al.*, 1981; Kostyo *et al.*, 1985). It also stimulates somatomedin production (Spencer *et al.*, 1981). Lactogenic effects of the 20K hGH are similar to those observed in the presence of the 22K compound in the pigeon crop-sac assay (Lewis *et al.*, 1978). On the other hand, the very marked decrease in the insulin-like activity was observed by Frigeri *et al.* (1979) using a purified preparation of 20K variant hGH, and by Kostyo *et al.* (1985) using a biosynthetic methionyl hGH-20K (Met-hGH 20K). Moreover, the latter have demonstrated the preservation of the chronic diabetogenic effect of this 20K variant of hGH as probably due to the insulin residual activity. It should be mentioned that the 20 and 22K forms of hGH represent an interesting model for studying structure—function relationships, since a deletion of 15 amino acids induces differences in biological activity.

Sigel *et al.* (1981) and Wohnlich and Moore (1982) have compared the biological activity by means of hormone-receptor binding. In a radioreceptor assay, where the 22K hGH is used as tracer, displacement by the 20K relative to the 22K

in the presence of liver membranes from pregnant doe rabbits, and between 22—53% in mammary gland membranes from pregnant doe rabbits. In contrast, Closset *et al.* (1983) and Smal (1986) reported a competitive capacity of the 20K hGH in the presence of the 22K hGH used as tracer ranging from 50—100% compared to the shift obtained with the 22K hGH in both receptor systems used (liver and mammary gland of pregnant doe rabbits). These results clearly show that the 20K hGH exhibits comparable behaviour to the 22K hGH towards these receptors. Purity and homogeneity of the 20 and 22K hGH preparations confirm these results. It may be concluded that normal hGH and its 20K variant have similar binding activities towards lactogenic or somatotropic receptors as well as identical biological activities *in vivo*.

In order to obtain further knowledge on the binding affinity of the 20 and 22K compounds for insulin-like receptors, Smal (1986) used hGH specific receptors isolated from cultured human lymphocytes (line IM-9) and rat adipocytes. Lymphocyte receptors similarly recognize the 22K form and the 20K variant with, nevertheless, a slightly lower affinity for the latter. However, the 20K functions as an agonist with a very low affinity ($\approx 3\%$) for rat adipocyte receptors as compared to the 22K. Thus, although the 20K hGH is not totally deprived *in vivo* of insulin activity (Frigeri *et al.*, 1979; Kostyo *et al.*, 1985), it possesses a very small intrinsic insulin activity *in vitro*. Thus, peptide 32—46 is most likely involved in the insulin activity; and it should be noted that the basic amino acid at position 41 is particularly conservative in each GH (Table I).

Placental growth hormones

hPGH. A placental human growth hormone has recently been noted by Hennen

et al. (1985) using monoclonal antibodies. This hormone exhibits an immunological cross-reaction with the 20K variant, but few data are as yet available to assess its structure-function relationships.

Placental hormones. Some placental hormones only have a lactogenic activity, such as human placental lactogen hormone (hPL), also called hCS (Florini *et al.*, 1966; Tsushima and Friesen, 1973), rat (Robertson and Friesen, 1975), mouse (Talamantes, 1975; Colosi *et al.*, 1982) and hamster (Kelly *et al.*, 1976; Southard *et al.*, 1986) placental lactogen hormones. Others have a lactogenic function associated with a significant somatotropic function, such as ovine (Chan *et al.*, 1976; Martal and Djiane, 1976; Martal, 1978), bovine (Fellows *et al.*, 1975; Bolander and Fellows, 1976), caprine (Becka *et al.*, 1977; Currie *et al.*, 1977), simian (Shome and Friesen, 1971), and guinea-pig (Kelly *et al.*, 1976) chorionic somatomammotropin hormones.

Unfortunately, only the sequence of placental hormones deprived of growth activity has been elucidated, such as hPL (Sherwood *et al.*, 1971; Li *et al.*, 1973) which exhibits an 85% identity and a 95% similarity with hGH, as well as rPL (Duckworth *et al.*, 1986) and mPL (Jackson *et al.*, 1986). The sequence of the latter two hormones was estimated from that of their complementary DNA. Analysis of these sequences showed a 50% homology of placental hormones with prolactins and 30% with growth hormones from corresponding species.

Although it has been established that placental hormones belong to the superfamily that includes PRLs and GHs, very little information is available on the sequence of amino acids constituting growth-promoting placental hormones, so that it is not possible to make structural comparisons.

Table II. (continued)

120	Glu	Glu	Gly	Ile	Gln Leu	Ala Thr	Leu	Met	Arg Gly Glu	Glu Arg	Leu	Glu	130
													Asp
	Gly Val	Ser Thr	Pro Ser	Arg	Ala Thr Val Ile	Gly	Gln	Ile	Leu Phe	Lys	Gln	Thr	Tyr
	Asp Ser	Lys	Phe	Asp	Thr Ala	Asn	Met Ser Leu	Arg His	Ser Asn	Asp	Asp	Ala	Leu
	Leu	Lys	Asn	Tyr	Gly	Leu	Leu	Ser Tyr	Cys	Phe	Arg Lys	Lys	Asp
170	Leu Met	His Asp	Lys	Ala Thr Ile Val	Glu	Thr	Tyr Phe	Leu	Arg Lys	Val Ile	Met Val	Lys Gln	Cys
	Arg	Arg Ser	Phe	Val Gly Ala	Glu	Ser Ala Gly	Ser	Cys	190	Phe-OH	Ala Gly		

* Gap

aa More present amino acid in GH molecules

[] Regions of Niall *et al.*

{ } Alternative sequences of Nicoll *et al.*

() Highly conserved domains of Kawauchi and Yasuda

() Amino acids numbering is based on primary sequence of hGH

Table III. Distribution of invariant regions, clusters and amino acids involved in GH activity.

Regions of internal homology in GH proposed by :			Clusters associated with GH activity (Nicoll <i>et al.</i> , 1986)		Basic amino acid related to GH bioactivity (Martal <i>et al.</i> , 1985)
Niall <i>et al.</i> (1971)	Nicoll <i>et al.</i> (1986)	Kawauchi Yasuda (1987)			
			G1 ^a	17-20	16
15-32	8-24	5-37		PG1 ^b	30-31
			G2	33-50	41
93-110	51-68	49-89		PG2	67-69
	69-86	104-126	G4	70-74	64
128-147	114-125		G5	106-113	70
				PG3	114-122
163-180	150-167	138-181	G6	130-158	77
					115
					134

G^a : GH binding determinants.

PG^b : PRL and GH binding determinants.

Prolactin-related proteins. Recently, Linzer and Nathans (1984) have identified in mouse placental tissue a prolactin-related glycoprotein exhibiting a growth-promoting activity. It was termed proliferin because of its stimulating effect on proliferation and differentiation of mouse cultured cells (Lee and Nathans, 1987).

Analysis of its primary structure showed a 31% and a 46% identity as compared to that of mouse and bovine prolactins, respectively. Moreover, it contained 2 tryptophan residues and 6 cysteine residues, as in most mammalian prolactins.

Molecular structure

It is interesting to determine whether secondary structures of growth hormones share a number of common features and to what extent they are related to somatotrophic function.

Disulfide bonds

Mills and Wilhelmi (1965) reported that after partial reduction of bGH (one disulfide bond out of two) using sodium sulfite, this hormone retained its biological potency *in vivo*.

Bewley *et al.* (1968) demonstrated that the existence of 2 hGH disulfide bonds is not essential for the expression of its biological activity. A marked somatotrophic activity *in vivo* has been observed by Li *et al.* (1977) with a recombination of 2 hGH fragments containing the NH₂-terminal part (1—134) non-covalently bound to the COOH-terminal part recovered after reduction of disulfide bonds, and blocking cysteine residues by carbamidomethylation.

Secondary structure

A method for predicting the secondary structures of proteins has been devised by Chou and Fasman (1978). Jibson and

Li (1979) used it for predicting the secondary structure of different human anti-pituitary hormones such as hGH. They reported an overall α -helix content for this hormone. Bewley and Li (1986), through studies on circular dichroism spectra, concluded that human and monkey growth hormones exhibited a similar α -helix content of $55 \pm 5\%$. The 3-dimensional structure of methionyl porcine somatotropin (MPS) has been determined by Abdel-Meguid *et al.* (1987) by use of the X-ray diffraction technique. It consists mainly of 4 antiparallel α -helices which account for 54% of the amino acid residues. The amino acid content within α -helices represents the predominantly invariant amino acid residues of other growth hormones. The hGH secondary structure has been comprehensively studied by Nicoll *et al.* (1986) and the different α -helix, β -turn, β -sheet and random coil forms have been quantified and positioned along exons II, III, IV, V. Recently, recombinant human growth hormone (r-hGH) has been crystallized by Jones *et al.* (1987).

Evaluation of the secondary structure is also compatible with the hydropathy profile obtained by the method of Kyte and Doolittle (1982). A hydropathy index takes into account hydrophilic and hydrophobic features of amino acid side-chains. These authors studied the tridimensional structures of some proteins by X-ray crystallography. There was a good correlation between the inner regions shown by the experiments and the hydrophobic areas on the one hand; and between the outer regions determined by the experiments and the hydrophilic areas on the other. Nicoll *et al.* (1986) examined the nature of the secondary structure and hydropathy profiles, studying either the hormone-specific regions or the regions with a high degree of amino acid homology. They observed a good correlation between

hydropathy profiles and secondary structures of regions with a high degree of homology. Nevertheless, although hormone-specific regions possess identical hydropathy profiles, they have noticeably different secondary structures. Thus, hGH and hPL, with nearly 95% sequence homology, exhibit very similar hydropathy profiles. The only differences concern 2 regions (59—67) and (100—117). The first one belongs to a region with common homology between GH and PRL and the second one is involved in a hormone-specific region (Fig. 3). Only one amino acid differs in the peptide 59—67 of hCS and hGH molecules located at position 64. hCS Methionine is replaced by a basic amino acid arginine in hGH. It should be pointed out that all the primary structures of GH studied (Table I) present a basic amino acid (arginine or lysine) at position 64; this fact suggests that this amino acid has a significant role in growth-promoting activity. The respective hCS (1) and hGH (2) sequences are the following from position 100 to 117 :

(1) Asn-Leu-Val-Tyr-Asp-Thr-Ser-Asp-Ser-Aps-Asp-Tyr-His-Leu-Leu-Lys-Asp-Leu

(2) Ser-Leu-Val-Tyr-Gly-Ala-Ser-Asn-Ser-Asp-Val-Tyr-Asp-Leu-Leu-Lys-Asp-Leu

Dissimilar amino acids at positions 104, 107, 110 and 112 correspond to noticeably different charges. All growth hormones (Table I) contain glycine at position 104, valine at position 110, glutamic or aspartic acid at position 112 like hCS. Thus, amino acids 104, 110 and 112 of hCS might explain the absence of growth activity in this molecule.

Structure of biologically active fragments of bGH has been defined by Chen and Sonenberg (1977); it was determined by methods of fluorescence spectroscopy and measurements of circular dichroism

spectra. Predictive analysis of helix, β -sheet and β -turn regions in bGH, according to these authors, is shown in Fig. 1.

The small biologically active peptide All (96-133) of bGH shifts from an α -helix to a random coil structure between pH 5 and 10.

Segments AI (1-95, 134-191) which have a low biological activity mainly exhibit a very rigid β -sheet structure. On the other hand, Chou and Fasman's prediction method determined a 45% α -helix structure in fragment AI1 and a 34% α -helix and a 22% β -sheet structure in fragment AI.

Studies on the secondary structure provided different results according to the methods used (CD spectra or Chou and Fasman prediction). The latter seems to be more reliable for studying native globular proteins at neutral pH. The method of Chen and Sonenberg is better adapted to the study of peptides in solution at different pH and ionic strengths.

Thus, there are discrepancies regarding the structural determinants of the biological activity of these proteins. However, the spatial proximity of lysine residues 70, 115 and 134 more particularly involved in somatotropic-like activity, as will be seen afterwards (Fig. 3), must be emphasized when analysing the representation of secondary structure of bovine growth hormone (Fig. 1).

Enzymatic hydrolysis

Numerous experiments have been carried out with GH submitted to proteolytic digestion in order to define biologically active sites.

The action of plasmin, thrombin, subtilisin and trypsin on the hGH molecule has been widely studied. hGH partial hydroly-

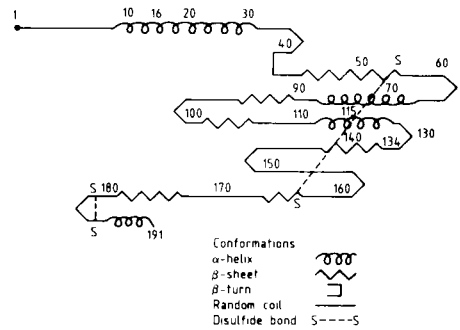


Fig. 1. Secondary structure of bGH predicted by the method of Chou and Fasman (according to Chen and Sonenberg, 1977). Lysines 70, 115 and arginine 134 involved in somatotropic activity

sis by trypsin does not affect its growth activity (Li and Samuelson, 1965).

After treatment of hGH with plasmin, Reagan *et al.* (1975) and Li and Bewley (1976) isolated 2 peptide fragments (1-134) and (141-191) linked only by a disulfide bridge between Cys 53-Cys 165. This new polypeptide retains a growth-promoting effect *in vivo* (weight gain and tibia tests in hypophysectomized rats) and a diabetogenic effect in the pancreatectomized rat similar to that of the native hormone. Moreover, only the NH_2 -terminal fragment (1-134) obtained after reduction and S-carbamidomethylation exhibits a low somatotropic activity *in vivo* (Reagan *et al.*, 1978).

The restrictive cleavage of hGH by thrombin at Arg-Thr bonds (134-135) was first observed by Li and Graf (1974), Graf *et al.* (1976) and confirmed by Mills *et al.* (1980). It gives rise to 2 peptides, 1-134 and 135-191, which after reduction and S-carbamidomethylation produce a new recombinant. The latter displays a significant activity in the rat tibia test.

Similar experiments have been performed by Reagan *et al.* (1981) on hGH after treatment with thrombin. The non-

covalent recombinant obtained between peptides 1—134 and 135—191 develops a noticeable growth activity estimated by the weight gain of hypophysectomized rats and equivalent to 35% of the native hGH activity. Its binding to liver plasma membranes of female rats only represents 20% compared to that of native hGH.

These lower activities may result either from structural modifications or from a different half-life as compared to that of native hGH in experiments *in vivo*. Attempts to recombine peptide 42—134 (obtained by digestion of peptide 1—134 by plasmin) either to peptide 135—191 or to peptide 141—191 were unable to induce a positive response in the rat weight gain test (Mills *et al.*, 1978).

More recently, Graf *et al.* (1982) using the limited action of trypsin on hGH did not observe any modification in the biological activity in spite of the excision of a segment of 11 amino acids at positions 135—145, during radioreceptor assays (on rabbit liver membranes) and tibia test in hypophysectomized rats.

The proteolytic action of subtilisin, similar to that of plasmin, gives rise to hGH molecules characterized by the deletion of amino acids 140—146 (Lewis *et al.*, 1977). Another modified form of hGH, deprived of residues 135—146, termed α_3 , has been reported by other authors (Singh *et al.*, 1974). These 2 forms of hGH exhibit a growth activity (evidenced by the tibia test) which is even higher than that of native hGH.

Analysis of the results regarding proteolytic effects on hGH indicates conservation of biological activity in most cases. It seems to be well established that residues 135—146 of hGH are not necessary to this activity.

In order to determine the distribution of one or more active sites of growth hor-

mones, some authors have used the recombinant method between different hormone molecules. Of particular interest for the study of structure—function relationships are the experiments of Burstein *et al.* (1978) and Russell *et al.* (1981). A hybrid is formed by the peptides obtained after plasmin action on hGH and hPL: an NH₂-terminal peptide of hGH (1—134) linked to a COOH-terminal peptide of hPL (141—191) by a disulfide bridge between Cys 53 and Cys 165. This hybrid has been tested for its binding capacity to mammary gland or liver receptors. This recombinant, whose NH₂-terminal structure corresponds to that of hGH, possesses the hGH activities, *i. e.* a binding capacity to somatotropic and lactogenic receptors and a secondary and tertiary structure similar to that of hGH as shown by circular dichroism spectra. On the other hand, the recombinant obtained by linking NH₂-terminal peptide of hPL (1—134) to the COOH-terminal peptide of hGH (141—191) does not display the somatotropic activity characteristic of the hGH molecule. An NH₂-terminal fragment of hGH (1—134) recombined to an hPL fragment (141—191) (Li, 1978) shows 50% growth activity *in vivo* (rat tibia test) as compared to an hGH recombinant composed of 1—134 and 141—191 parts obtained under the same conditions, *i. e.* non-covalently linked (after reduction and S-carbamidomethylation of cysteine residues) (Li *et al.*, 1977) (Fig. 2). This decreased activity seems to be attributable to the existence of 2 different amino acids between hPL and hGH and to be located in the last third of the molecule. Histidine 153 and methionine 179 of hPL replace aspartic acid and isoleucine from hGH, respectively. Isoleucine (179) and methionine (179) are both hydrophobic amino acids, whereas basic histidine (153) and aspartic acid (153) present a different charge. This difference might

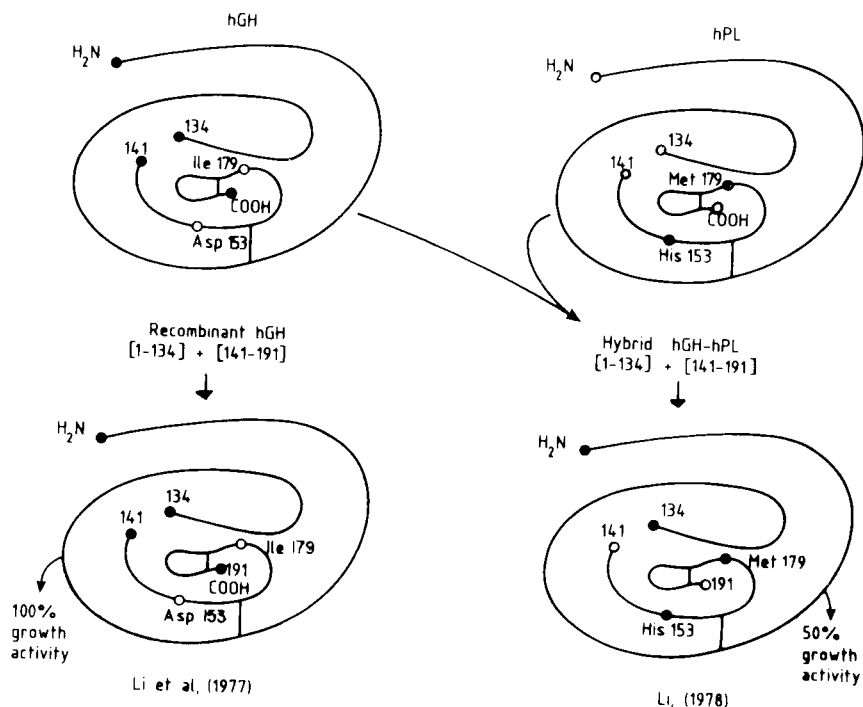


Fig. 2. Evidence for one acidic amino acid in hPL inhibiting growth bioactivity (measured by rat tibia test) and related to hGH and hPL peptide recombinants.

explain the marked inhibition of the hybrid hormone hGH—hPL growth activity.

Therefore, the somatotropic and structural properties of hGH are related to the NH₂-terminal part of the hormone, in particular peptide 1—134.

Graf and Li (1974b) and Yamasaki and Shimanaka (1975) have shown that a fragment composed of 38 amino acid residues (96—133) isolated from bGH or oGH following restricted trypsin hydrolysis exhibits significant growth activity *in vivo*.

Thrombin action on oGH is characterized by a cleavage between residues 133 and 134 (Arg-Ala). Two peptides (1—133 and 134—191) are obtained. According to the results of Graf *et al.* (1976), these peptides are totally inactive in the rat tibia

test, whereas peptide 96—133 of oGH obtained by trypsin retains its biological potency in the same test. It seems that thrombin action more drastically modifies the tertiary structure of oGH than trypsin and produces different metabolically active fragments.

Inactivity of peptide 1—133 of oGH seems to contradict the previous experiments of Li and Graf (1974). Using hGH plasmin digests, these authors observed that only the 1—134 NH₂-terminal peptide manifested a 10—20% biological activity *in vivo* as compared to native hGH. These differences in the activity of NH₂-terminal fragments of oGH and hGH are probably due to differences in the structure of the peptides obtained from human and rumi-

nant (ovine and bovine) GHs (Bewley and Li, 1972; Li, 1972).

Li (1975) has attempted to localize more accurately the biological activity site in peptide 1—134 of hGH. Cyanogen bromide gives rise to peptide 15—125 devoid of secondary and tertiary structure, and which nevertheless retains a growth-promoting activity in the rat tibia test as well as a lactogenic activity in the pigeon crop-sac assay. Thus, part 15—125 of hGH molecule retains most of the biological activities.

Liberti (1981) observed a small somatomedin-like activity due to 87—124 peptide of bGH, as shown by the stimulation of sulfate and thymidine incorporation by the costal cartilage of the hypophysectomized rat, while native bGH is devoid of this activity. Liberti and Durham (1983) have obtained 1—133 peptide after thrombin action on bGH. This peptide exhibits a marked somatomedin-like activity similar to that of peptide 96—133 (Liberti and Miller, 1978). These experiments clearly suggest that residues 96—124 are essential for the expression of the somatomedin activity. Nevertheless, Li and Bewley (1976), and Reagan *et al.* (1979) have demonstrated that total expression of growth-promoting activity requires the presence of the COOH-terminal peptide.

Synthetic peptides

Very few experiments have been performed on hGH synthetic peptides. Chillemi and Pecile (1971) have measured *in vivo* the biological activity of 2 synthetic peptides obtained by Merrifield's technique (1969) (a monotetracontapeptide 81—121 and a ditriacontapeptide 122—153). Each peptide provokes the thickening of the rat tibia cartilage. This test also

shows a remarkable synergy of action between both polypeptides. Nevertheless, the potency of these synthetic peptides remains very low, which emphasizes the role of the tertiary structures conservation of growth hormones.

Blake and Li (1973) have reported a growth-promoting activity of a synthetic peptide : N- α -acetyl-hGH (95—136) detected by the rat tibia test. Another localization of somatotropic activity has been demonstrated by Retegui *et al.* (1982), using monoclonal antibodies specific for 3 hGH synthetic peptides: 19—128, 73—128, 98—128. They investigated the interference of these different anticlonal antibodies with the binding of hGH to its receptors. The highest inhibition of hGH binding to receptors occurs when using the antibody directed against the 98—128 peptide.

Results of this work suggest that the hGH binding site to liver receptors is located in the NH₂-terminal part of the hormone and in particular at positions 98—128.

A growth-promoting activity test more sensitive although more partial than the conventional test on the rat tibia was used by Morikawa *et al.* (1984). It measures the effect of 2 synthetic peptides of hGH, 1—43 and 32—46, on the conversion of 3T3 preadipocytes into adipose cells. It does not reveal any activity, whereas we noticed the important insulin-like effect of peptide 32—46 of the 20K hGH-variant binding to adipocytes. According to these results, the whole molecule does not seem necessary for the expression of any growth-promoting activity.

Finally, localization of the sites responsible for somatotropic activity, in particular that of hGH, has been defined as follows: a global localization in the NH₂-terminal part (1—134 peptide) on the one hand,

and a more restricted localization in peptides 15—125, 81—121 or 122—153 or 95—136 on the other. Moreover, the binding capacity of hGH to liver receptors seems to be more particularly supported by peptide 98—128.

The hypoglycemic action of growth hormone has been reported by Milman and Russell (1950). Ng *et al.* (1974) first attributed the insulin-like effects to the amino-terminal (1—15) region of the hGH molecule. In particular, the synthetic human growth hormone fragment (hGH 4—15) stimulates *in vivo* and *in vitro* 2-deoxyglucose uptake in rat adipocytes (Ng and Harcourt, 1986). These authors explain the hypoglycemic action of hGH by an interaction of the fragment 4—15 with the plasma membranes of the target cells (adipocytes and hepatocytes) inducing the release of a cellular mediator which would enhance insulin binding and hexose transport (Ng *et al.*, 1985). A longer peptide, hGH 1—43, was found to occur naturally in pituitary extracts (Singh *et al.*, 1983) in significant amounts (at least 12 µg/gland) (Frigeri *et al.*, 1988). These authors partially reinforce the hypothesis of Ng *et al.* in showing that this fragment hGH 1—43 is able to enhance the *in vitro* sensitivity of adipose tissue of yellow obese mice to insulin action. Insulin-stimulated glucose oxidation was enhanced over 100% without serum insulin concentrations being increased.

Let us recall that 20K hGH, which lacks fragment 32—46, exhibits little or no early insulin-like actions (Frigeri *et al.*, 1979; Smal *et al.*, 1986). One implication of these observations is that most of the insulin-like properties of the native hormone could reside in the deletion peptide. Stevenson *et al.* (1987) tested this deletion peptide hGH 32—46 in the conscious dog and found that it increased glucose-induced insulin secretion, which in turn

enhanced tissue uptake of glucose. Rudman and Stebbing (cited by Stevenson *et al.*, 1988) showed in the rat that the smaller peptide hGH 32—38 is even several-fold more active than the deletion peptide itself.

Thus, it appears that insulin-like properties of GH are limited to the N-terminal sequence of the molecule. However, the data are not clear enough to attribute a pure function to a cluster of few amino acids, since the synthetic hGH 1—43 is more potent than any of the shorter peptides studied in enhancing insulin-stimulated action.

Chemical modifications

Reduction of disulfide bonds

This reduction followed by alkylation of SH groups of a growth-promoting hormone does not modify its biological activity, as shown by Li and Bewley (1976) on a plasmin-treated hGH. The binding capacity to liver membranes of other growth hormones has been estimated after submitting the S-S bridges to sodium borohydride. In these experiments, the bindings of hGH, oCS (ovine chorionic somatomammotropin or ovine placental lactogen) and bGH were almost similar to those of their respective native hormones (Chêne *et al.*, 1984; Martal *et al.*, 1985).

A partial reduction of bGH in the presence of dithiothreitol followed by iodoacetamide alkylation of cysteine residues 182 and 189 did not affect its growth activity (Graf *et al.*, 1975).

Punctual chemical modifications

Punctual chemical modifications have been applied to some amino acids. An example of this is the work of Cascone *et*

al. (1980) on oxidation of methionine at positions 4, 125, 150 and 180 of bGH. These authors did not report any reduction in the growth activity of this modified hormone in the body weight gain test of hypophysectomized rats.

In contrast, nitration of 6 tyrosine residues out of the 8 present in hGH using tetranitromethane leads to a reduction of $\approx 60\%$ in the *in vivo* growth activity compared to native hGH. Moreover, the same reaction in the presence of guanidine-HCl 5M provokes the nitration of 8 tyrosine residues and the total loss of somatotrophic activity of this modified hormone (Ma *et al.*, 1971). This inactivation may be attributed to structural changes in the hormone caused by nitration. In fact, the structure of the nitrated molecule is far less rigid than that of the native hormone, as shown by its higher sensitivity to tryptic digestion.

Nitration of some tyrosine residues was performed on equine and bovine GHs by Daurat-Larroque *et al.* (1977). The reactivity of the different tyrosines towards tetranitromethane is variable. The most sensitive are tyrosine residues 35 and 176 for bGH and 35 for eGH, followed by tyrosine 42 for both hormones. Residues 111 for bGH and 111, 143 and 176 for eGH react partially, while tyrosines 143 and 160 for bGH and 28 and 160 for eGH remain unchanged. In these conditions, nitration also affects the tryptophan residue of both hormones. The growth activity estimated by the weight gain test represents 100% for nitrated-bGH and 84% for nitrated-eGH compared to native hormones, respectively. On the other hand, nitration of bGH and eGH, under denaturing conditions (in the presence of 8M urea) totally inhibits the biological activity of both hormones. A change in the structure of these hormones may account for this loss of activity, but it has not been proved. These authors concluded that tyrosine residues 35, 42, 111 and 176 and

tryptophan 86 for bGH and eGH and tyrosine residue 143 of eGH do not affect the biological activity of these hormones in non-denaturing conditions.

Acetylation of bGH by N-acetylimidazole of the following tyrosine groups given by order of decreasing reactivity: Tyr 42, 160, 35, 111, 143 and 176, is also accompanied by acetylation of some lysine residues (Blumgrund de Satz and Santomé, 1981). In these conditions, bGH exhibits *in vivo* a small growth activity and is not able to compete with iodinated bovine growth hormone in the binding to liver membranes, although modified bGH retains its α -helix structure. Growth activity of bGH *in vivo* is maintained when amidination of lysine residues is performed in parallel with acetylation of tyrosine residues. These results are in agreement with those of Daurat-Larroque *et al.* (1977). They indicate that first tyrosine residues are not involved in growth activity. Iodination of 3 tyrosine residues of bGH does not interfere with the structure of this hormone, which exhibits a similar circular dichroism spectrum (Mattera *et al.*, 1982) and does not modify its activity for somatotrophic sites (Mattera and Dellacha, 1982). Biscoglio de Jimenez Bonino *et al.* (1979) have observed a 50% reduction *in vivo* in the bGH growth activity after trinitrophenylation of lysines 181, 145, 70, 113, 172 and 168 (residue 158 is excluded in this reaction), without change in the α -helix structures suggesting that lysines are implicated in the expression of this activity through the positive charges carried by lysine.

Different alkylation reactions (methylation and ethylation) as well as guanidination and acetimidation have been performed on lysine residues of 3 growth-promoting hormones such as bGH, hGH and oCS (Chêne *et al.*, 1984; Martal *et*

al., 1985). They showed that whatever the degree and kind of chemical modification, lysine residues participate in the hormone-hepatic receptor interaction. Among them may be mentioned the following basic amino acid residues (lysine or arginine) at positions 16, 41, 64, 70, 77 and 134 as well as lysine 115, highly conserved in prolactins, and which are liable to be involved in one or several sites of recognition of somatotropic receptors (Chêne, 1987).

In conclusion, among the numerous chemical modifications performed up to now, only those affecting lysine have been found to have a negative effect on the *in vivo* biological activity and on the binding capacity to the liver receptors of GHs.

Nicoll *et al.* (1986) have identified several G clusters specifically associated with GH activity (binding affinity and *in vivo* potency), and PG clusters connected with both GH and PRL activities, by analysis of predicted hydropathy profiles combined with the secondary structure of consensus GH, hGH and pPRL (porcine prolactin). Among clusters located in the 1-134 fragment molecule there are clusters G (G1-G2-G4-G5-G6) at the following amino acid positions: 17-20, 33-50, 70-74, 106-113 and 130-158 (Table III). Moreover, clusters PG1 (30-31), PG2 (67-69), PG3 (114-122) characterize common binding determinants for GH and PRL.

Among the basic amino acids studied by Chêne *et al.* (1984) and Martal *et al.* (1985) and liable to participate in the growth hormone-receptor relationship, most are included or closely related to the binding determinant clusters identified by Nicoll *et al.* (1986) (Fig. 3). Consequently, arginine at positions 64 and 77 of hGH seems to be excluded from a binding site to somatotropic receptors. Moreover, Lys

41 is not essential for hGH binding to somatotropic receptors, since the 20K variant of hGH, lacking 32 to 46 amino acids, binds both lactogenic and somatotropic receptors (Fig. 3; Table III). Clusters identified by Nicoll *et al.* (1986) and basic amino acids selected by Martal *et al.* (1985) and involved in a somatotropic-like activity do not correspond precisely with the invariant sequences proposed by Niall *et al.* (1971), Nicoll *et al.* (1986) and Kawauchi and Yasuda (1987). The expected overlap between invariant and biologically active areas of GH could not be clearly verified (*cf.* Table III).

Genomic modifications

hGH genes

Two genes coding for hGH have been identified (Fiddes *et al.*, 1979; Seeburg, 1982). hGH-N gene encodes for the

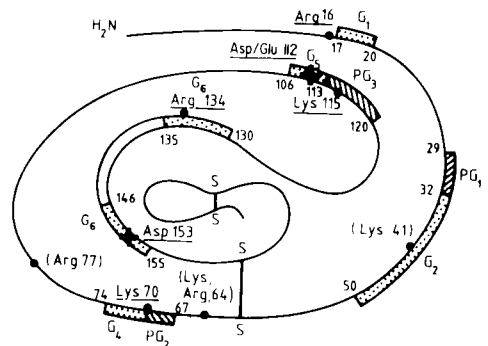


Fig. 3. Model to localize biological sites in growth hormones. Somatotropic activity regions of GH molecules (according to Nicoll *et al.* 1986) and localization of basic amino acids (according to Martal *et al.*, 1985). : G Growth hormone binding determinants; : PG Prolactin and growth hormone binding determinants; aa : Basic amino acids involved in growth activity; + : Aspartic or glutamic acid involved in growth activity. Inactive peptide 135-146 (according to Graf *et al.*, 1982)

sequence of the major form of hGH, the 22K, as well as the subsequent forms that are derived from it (see "Growth Hormone. I. Polymorphism"; Charrier and Martal, 1988) including the 20K variant from an alternative splicing of premessenger RNA (Wallis, 1980; De Noto *et al.*, 1981), and a new natural 17.5K variant (Lecomte *et al.*, 1987). The 20K variant represents the product of N gene in which the second intron has been extended at the expense of part of the exon encoding for the 22K hGH sequence.

It has been previously demonstrated that deletion of amino acids 32—46 led to the almost total loss (80%) of insulin-like activity of the 20K as compared to the 22K (Frigeri *et al.*, 1979; Kostyo *et al.*, 1985). The second hGH-V gene seems to encode for a polypeptide of 191 amino acids experimentally transcribed *in vitro*, in which 13 amino acids differ from the 22K of hGH (Pavlakakis *et al.*, 1981). It has been verified that V gene encodes for placental hGH (Hennen *et al.*, 1985; Franckenne *et al.*, 1987) and the "invisible" hGH (Bistrizter *et al.*, 1988).

Methionyl-GH

A methionyl-hGH synthesized by *E. coli* has been purified by Olson *et al.* (1981). They demonstrated that natural hGH and the Met-hGH derivative obtained by genetic engineering are similar in both weight gain and tibia tests in hypophysectomized rats. The same growth activity has been described by Kostyo *et al.* (1985) for the 20,000-dalton Met-hGH.

A Met-bGH exhibits a growth activity of 1.4 U/mg detectable by the increased weight gain of hypopituitary-dwarf-mouse *versus* 1 U/mg for a natural pituitary bGH (Hart *et al.*, 1984).

Accordingly, growth activity is not affected when growth hormones are bio-

synthesized, *i.e.*, obtained with or without supplementary methionine at the NH₂-terminal (Moore *et al.*, 1988).

Conclusion

Structure-function relationships of growth hormones, especially hGH and bGH, were mainly studied with reference to growth activities *in vivo* and binding to somatotrophic receptors. The data obtained will contribute to a better fundamental knowledge of biologically active sites of this GH family. However, some results are still inconsistent.

Thus, although the gene reduplication of the common ancestral peptide seems to be highly probable between the different GH and PRL families, determination of peptide sequences corresponding to internal homologies between these molecules is still uncertain and variable.

Estimation of the secondary structures of hGH, bGH, oGH and mGH, which requires the use of different methods, is still restricted. Accordingly, it is not possible to specify a relationship between secondary and tertiary structures and somatotrophic function. Clearly the presence of two disulfide bonds is not implicated in the expression of its biological activity. On the other hand, it has been well established that the first two-thirds of the hGH molecule (peptide 1—134) display the specific biological activity, although the full expression of the latter requires the presence of the last third (peptide 141—191). So, the whole molecule would maintain the correct structure of one or more restricted binding sites. The fragment 135—145 does not contribute to the somatotrophic activity. The involvement in the insulin-like activity of peptide 32—46, missing in the 20K hGH, is most probable as is also peptide 4—15 of hGH.

The somatomedin-like activity is attributed more particularly to fragment 96—124 of bGH. The same part of the hGH molecule (98—128) intervenes in growth activity *in vivo*, as well as in highly specific binding to liver membranes. A marked decrease in the growth activity of hGH (1—134) and hPL (141—191) recombinant might be mediated by the existence in hPL of histidine replaced by aspartic acid in hGH at position 153, suggesting that the latter residue could be important for the growth hormone activities of some placental lactogens such as ruminant CS. In the same manner, hPL exhibits a methionine residue at position 64 instead of Arg or Lys in every molecule of GH.

In addition, hPL displays an aspartic acid residue at position 104 *versus* glycine residue in every GH, an aspartic acid residue at position 110 *versus* valine residue in every GH and a histidine residue at position 112 *versus* an aspartic or glutamic acid residue in every GH studied.

The integrity of some amino acids, particularly lysines, is essential for the full expression of the biological activity. It may be asserted that lysine or arginine at positions 16, 70, 115 and 134 is involved in the growth hormone-receptor binding.

In the future, directed mutagenesis studies should lead to a more accurate definition of the structure—function relationships of growth-promoting hormones.

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