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Journal of Chromatography B, 688 (1997) 161–177

JOURNAL OF CHROMATOGRAPHY B

Review

Structure and function of bovine growth hormone Bovine growth hormone as an experimental model for studies of protein–protein interactions

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Received 16 April 1996; revised 18 June 1996; accepted 27 June 1996

Abstract

Growth hormone (GH) is a polipeptide that controls the differentiation, growth and metabolism of many cell types, and is secreted from the hypophysis of all vertebrate species tested so far. Despite the overlapping evolutionary, structural, immunological and biological properties, it is well-known that GHs from distinct mammalian species have significant species-specific characteristics. The main purpose of this review is to highlight bovine GH (bGH) structural features related to its species-specific properties. Novel interest in bGH is also aroused by the advent of biotechnological methods for production of recombinant proteins. In fact recombinant bGH will have a great importance in veterinary medicine research and as a 'high tech' drug that needs to be monitored in zootechnical productions.

Keywords: Reviews; Growth hormone; Bovine growth hormone; Proteins

Contents

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PII S0378-4347(96)00296-4

1. Introduction

Growth hormone (GH) has numerous metabolic actions on many cell types, and generally is responsible for tissue and proportionate somatic growth. Only recently have significant details emerged concerning the molecular basis of these actions. This review will assess current understanding of the structure and, where possible, provide information on structure–function relationships of bovine GH (bGH).

The importance of GH in the control of growth and reproduction of cattle, and the recent use of recombinant bGH to boost livestock production, have been the chief stimuli to studying which of the hormone's structural features are related to its biological effects. Interest in GH from domestic animals is also aroused by evidence that animal GH may in some ways parallel the human hormone (hGH). In fact, despite considerable species specificity, GHs from different species share many biological, immunological and structural features. The bovine species has proved to be an especially interesting animal model for studying gene expression and the evolution of secretion mechanisms of this classical growth promoting hormone.

2. Structural studies of bGH

GH is a single-chain polypeptide hormone synthesised and secreted by the pituitary gland under hypothalamic control. It belongs to the same hormonal family as prolactin (PRL) and placental lactogen (PL), all of them sharing certain biological, immunological and structural features; they are considered to be derived from duplication of a single ancestral gene [1–3]. These three hormones, and some non-pituitary hormones such as interleukins 1–7, granulocyte–macrophage colony stimulating factors (GM-CSF) and erythropoietin (EPO), belong to a larger and growing super-family, labelled the hematopoietic superfamily. The members of this family have the same overall three dimensional structure, but often share little or no sequence similarity [4].

Li first isolated the bGH from pituitary glands in 1944 [5]. Bovine GH is a protein of 191 amino acids, with a molecular mass of 21 819, as calculated from the amino acid sequence. The molecule has two disulphide bonds at 53–164 and 181–189 positions (Fig. 1). As shown in Fig. 2, the mobility in SDS electrophoresis of bGH is related to the reduced or oxidised state of its disulphide bridges (Secchi, unpublished data): in its S–S bonded form, bGH has a more compact structure which results in a lower apparent molecular mass.

Sequence data for GH are now available either from direct protein sequencing or from the sequence of cloned cDNA from a considerable number of

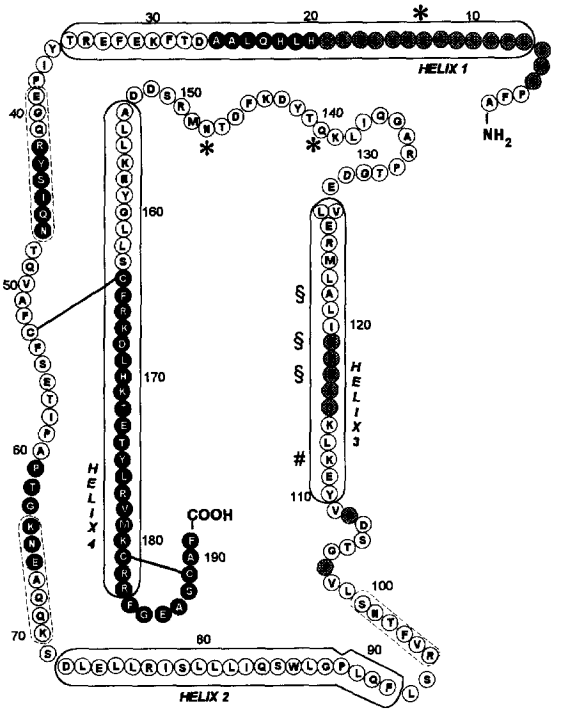

Fig. 1. Schematic representation of the bovine growth hormone molecule. Each circle represents an amino acid residue. The 'sausages' show the four helix bundles (helix 1: L7–T35; helix 2: D72–F92; helix 3: Y110–V/L127; helix 4: A154–R183). The dashed lines indicate the position of possible mini-helices (E39–N47; K64–K70; R95–S100). The heterogeneity at residues 1 and 127 is discussed in the text. Cystine bridges have been assigned at residues 53–164 and 181–189. Asterisks (*) mark the amide residues susceptible to deamidation at residues 13, 140, and 148. Filled circles indicate the probable residues involved in binding with the dimeric receptor (black, site I; grey, site II). Residue K112L is Brems's mutant (≠) [38], and residues E117L, G119R and A122D are Chen's mutants (§) [37].

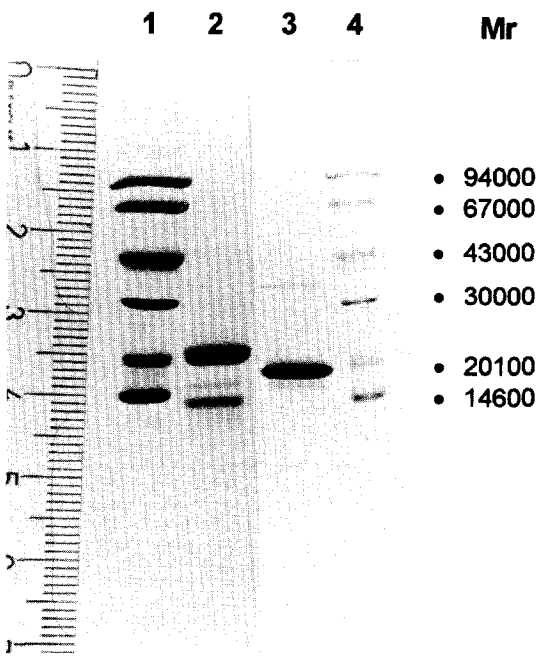

Fig. 2. SDS polyacrylamide gradient gel electrophoresis of bGH under reducing and non reducing conditions. SDS electrophoresis was performed in a polyacrylamide gradient gel (7–20%) overlaid with 4% stacking gel using a Minigel apparatus (Biometra, Gottingen, Germany), at constant current of 10 mA for 15 min and then at constant voltage (170 V) for about 1 h. The gel was stained with silver nitrate. Lane 1: standard proteins for molecular mass. Lane 2: 2 micrograms bGH dissolved in the presence of 5% of 2-mercaptoethanol. Lane 3: 2 micrograms bGH in the unreduced form. Lane 4: standard proteins for molecular mass. The molecular mass (M_r) of standard proteins is listed on the right.

species, permitting detailed analysis of sequence homologies. In Fig. 3 the bGH amino acid sequence is compared to the GHs from 18 mammals, two birds, two reptiles and eight fish [2,3,6–32]. Alignment of the protein sequence was straightforward, and based on that used previously [3]. Pair-wise comparisons (bovine vs. other species) were made for each type of amino acid sequence (Table 1). The sequences of sheep and goat GH are identical and differ from bGH at two residues. The sequences of any of these differ from the other terrestrial non-primate mammalian sequences by 18–24 residues, and from primate GH by 64 residues. Differences from birds and fish are larger, reaching 122 different residues for bonito.

2.1. bGH heterogeneity

Even when highly purified, bGH always presents a complex heterogeneity with different molecular forms. This is partly due to naturally occurring genomic transcripional variants and translational modifications. The presence in fresh tissue as well as in hormone fractions purified under mild conditions (for a review of bGH purification procedures allowing the highest recovery of specific biological activity, see Bell et al. [32]), or by one-step immunoaffinity purification [33], suggests a physiological significance for many of the components. In particular, the use of immunoaffinity chromatography to purify proteins implies one single requirement, namely the occurrence of the recognised epitope(s) on the molecule to be purified. An immuno-affinity-purified product might therefore be more complex than the product obtained by a more conventional purification procedure, wich can isolate isoforms of different charge, size and solubility. The availability of monoclonal antibodies highly specific for bGH makes the use of immunosorbent chromatography feasible as a means of preparing milligram quantities of hormone suitable for a successive characterisation. Fig. 4 shows the silver-stained SDS electrophoresis pattern of a one-step purification procedure of bGH by an anti bGH–MAb (MAb 5G1 [34]) coupled to solid phase (Secchi and Borromeo, unpublished results). The epitope recognised by the MAb used is conformation dependent and has made this MAb suitable for affinity purification of bGH in its native active form. A shift to pH 3.5 was sufficient to promote dissociation of the antibody antigen complex and the immediate raise to pH 8.5 after the elution restored the stability of bGH.

Individual members of this structurally heterogeneous peptide family of bGH might have different effects on target organs, either by interacting differently with a single class of receptor, or by binding preferentially to particular types of receptors which mediate specific actions [35].

2.1.1. Transcriptional variants

Natural bGH. The transcriptional variant in bGH is known where both V and L are found in a ratio of 1:2 at residue 127. As demonstrated by Seavey et al. [36]

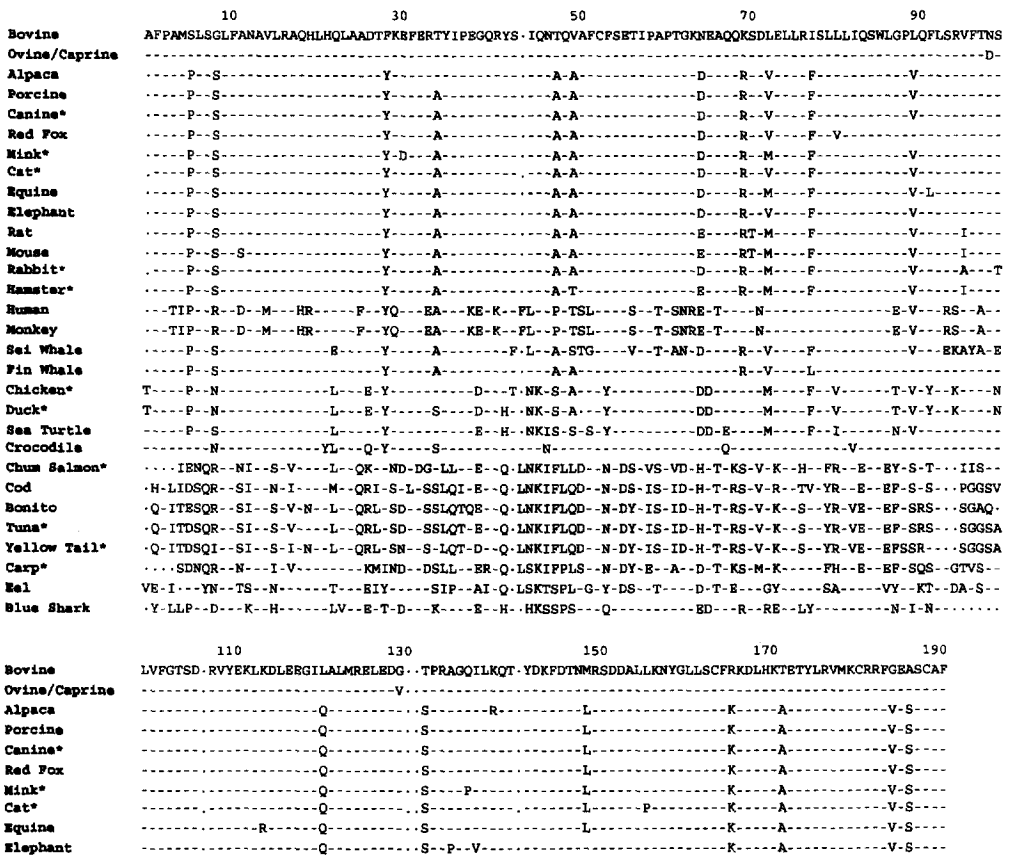

Fig. 3. Allignment and comparison of amino acid sequences for GHs from 18 mammals, two birds, two reptil and eight fish. The sequences are compared with that from the bovine species. - indicates identity, · indicates a gap. The sequences marked with (*) are from the cDNA. Data for sequences are from the following references: bovine (Bos taurus) [6]; ovine (Ovis aries) and caprine (Capra hircus) [7]; alpaca (Lama pacos) [8]; porcine (Sus scrofa) [9]; canine (Canis familiaris) [10]; red fox (Vulpes vulpes) [3]; mink (Mustela vison) [11]; cat (Felis catus) [12]; equine (Equus caballus) and rat (Rattus norvegicus) [3]; elephant (Loxodonta africana) [13]; mouse (Mus musculus) [14]; rabbit (Oryctolagus cuniculus) [15]; hamster (Mesocricetus auratus) [16]; human (Homo sapiens) [2]; monkey (Macaca mulatta) [17]; sei whale (Balenoptera borealis) [18]; fin whale (Balenoptera physalus) [19]; chicken (Gallus gallus) [20]; duck (Anas platyrhynchos variant domesticus x Cairina moschata) [21]; sea turtle (Chelonia mydas) [22]; crocodile (Crocodylus novaeguineae) [23]; chum salmon (Oncorhynchus keta) [24]; cod (Gadus morhua) [25]; bonito (Katsuwonus pelamis) [26]; tuna (Thunnus thynnus) [27]; yellow tail (Seriola guingueqadiata) [28]; carp (Cyprinus carpio) [29]; eel (Anguilla japonica) [30]; blue shark (Prionace glauca) [31].

Table 1
Sequence homology between bGH and GHs from other species

Species	Number of amino acids identical to bGH	Percentage homology
Ovine/caprine	189	99.0
Alpaca	173	90.6
Porcine	173	90.6
Canine	173	90.6
Red fox	173	90.6
Mink	171	89.5
Cat	172	90.1
Equine	171	89.5
Elephant	172	90.1
Rat	168	88.0
Mouse	167	87.4
Rabbit	167	87.4
Hamster	170	89.0
Human	127	66.5
Monkey	127	66.5
Sei whale	159	83.2
Fin whale	176	92.1
Chicken	145	75.9
Duck	146	76.4
Sea turtle	149	78.0
Crocodile	148	77.0
Chum salmon	76	39.8
Cod	70	36.6
Bonito	69	36.1
Tuna	70	36.6
Yellow tail	70	36.6
Carp	79	41.4
Eel	101	52.9
Blue shark	113	59.2

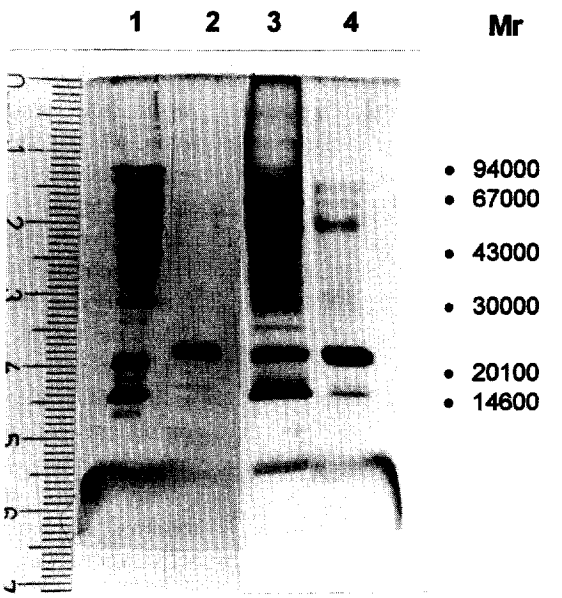

Fig. 4. SDS electrophoresis of bGH purified by immunoaffinity chromatography. The immunosorbent matrix was obtained by coupling 7 mg of an anti-bGH MAb (MAb-5G1) per ml of divinilsulfone activated agarose (Mini Leak, Kem-En-Tec, Hellerup, Denmark) according to the manufacturer's instructions. Crude pituitary extract (approx. 10 mg total proteins) diluted in phosphate buffered saline (PBS) was loaded. The column was washed with starting buffer containing 300 m*M* NaCl and 0.1% (v/v) Tween 20. Bound material was eluted with a glycine–HCl buffer (50 m*M*, pH 2.5). The electrophoresis was performed as described in Fig. 2. Lane 1: standards for molecular mass. Lane 2: 2 μg bGH standard. Lane 3: 10 μm pituitary extract. Lane 4: 2 μm immunoaffinity prepared bGH. The molecular mass (M_r) of standard proteins is listed on the right.

the variation at this position is due to an allelic polymorphism.

Genetically engineered bGH. Apart from the natural hormone (bGH NH_2–A–F–), several biosynthetic bGHs with distinct N-terminal variants are now produced by different firms: (1) NH_2–M–F– by Monsanto, (2) NH_2–M–D–Q–F– by Cyanamid, (3) NH_2–M–F–P–L–D–D–D–D–D–K–F– by Elanco, 4) NH_2–M–F–P–L–D–D–D–D–K–F– by Eli Lilly. Although produced in bacteria or in eukaryotic cells (or hypophyseal cells), they have full biological activity in vivo and can be considered true variants of the natural hormone. They are believed to be more immunogenic, but antibody titres are only rarely sufficiently high to interfere with bGH bioactivity in vivo.

A mutant bovine GH with three amino acid substitutions in helix 3 (E117L; G119R; A122D) (Fig. 1), designed to increase its amphiphilicity, was reported to retain full binding affinity to GH receptors in mouse liver membranes. However, transgenic mice containing the mutated bGH gene showed a significant growth-suppressed phenotype, the degree of suppression being directly related to serum levels of altered bGH molecule. An uncoupling of the growth-promoting and receptor-binding activities of GH has been suggested. This mutant therefore appears to act as a GH antagonist [37].

In line with the general strategy of oligonucleotide-directed site-specific mutagenesis the change K112L was encoded in bGH molecule, and its effect on folding was studied [38]. The mutant protein refolds more slowly and precipitates more readily on refolding than the wild type.

2.1.2. Translational modifications

Among various co-translational modifications bGH presents two aminoacyl N-terminal residues, F and A, in approximately equimolar quantities, and a third one present with less frequency, M. All these modifications seem to arise from processing of the growth hormone precursor. The A form can be separated from the other two by preparative isoelectric focusing (IEF) of purified bGH preparations. All chains have equipotent biological activity, as measured by tibial assay [39]. Fig. 5 shows the results of an analytical IEF on polyacrylamide gel of these isoforms recovered by preparative IEF of pituitary derived bGH [40]. The two components of the starting material (lane 1), are recovered in two fractions, namely the cathodic (lane 2) and the anodic band (lane 3). By N-terminal sequential analysis 93% A and 7% N-terminal F were found in sample 2, while 48% F, 30% A and 22% N-terminal M were found in sample 3. The phenylalanyl and methionyl chains can not be separated by IEF because they have the same p*I*. The N-terminal M form is supposed to be derived from the proteolytic cleavage of the N-terminal F chain [39].

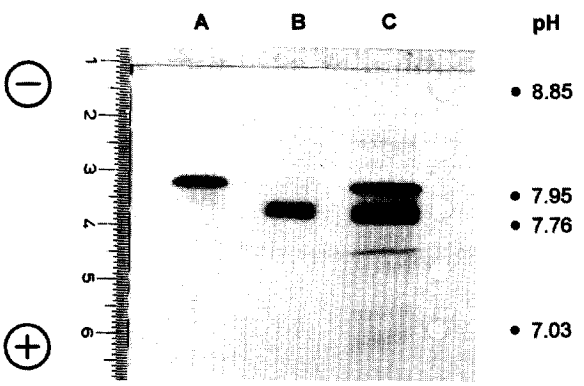

Fig. 5. Analytical isoelectric focusing in polyacrylamide gel (PAGIF) of distinct N-terminal forms of bGH. PAGIF was performed in 5% polyacrylamide gel (0.4×100×120 mm) with 2% Ampholine, pH 3–11 range, at 4°C using LKB 2117-010 Multiphor equipment. PAGIF was carried out at 5 W/gel. The cathodic and anodic electrolytes were solutions of 1 *M* NaOH and 1 *M* H_3PO_4, respectively. Samples were dissolved in 1% Ampholine pH 9–11. The gel was stained by silver nitrate. Lane 1: pituitary purified bGH. Lane 2: bGH A N-terminal form. Lane 3: bGH F N-terminal form. The pH scale and the p*I* values of the bands (7.95–7.76) are listed on the right. Reprinted from Ref. [40] with permission.

Post-translational modifications that might play a physiological role include the deamidation of asparaginyl (N) and glutamyl (Q) residues to aspartic (D) and glutamic (E) acid residues. The N and Q residues of bGH susceptible to deamidation were identified (positions 13, 140 and 148) by amino acid sequencing of the tryptic peptides separated by reversed phase HPLC [41]. Fig. 6 shows the peptide mapping of the trypsinised bGH. Peptides eluted under each peak were designated by arabic numerals starting from the N-terminus of the primary sequence. All peaks (with the exception of T4 and T14 that were successively separated by rechromatography), contained a single tryptic fragment. Peptides T1 (sequence 1–17), T13 (sequence 126–133) and T14–15 (sequence 140–150) gave more than one peak. T1 eluted at 42, 45, 48 and 49 min. Sequence analysis showed that peaks at 42 and 45 min were the completely amidated forms of the A and F N-terminal peptides (discussed in the previous paragraph), while peaks eluted at 48 and 49 min were the A and F peptides containing D13 (instead of N). Therefore, deamidation at N13 can take place in both the N-terminal forms of bGH. T14–15 (despite the presence of K144, residues 140–150 were exclusively recovered as a unique fragment in the experimental conditions used) eluted in two peaks, the first (24 min) contained the completely amidated form, while the same fragment containing E140 and D148 was found in a second peak with retention at 127 min. It has been demonstrated that the two T13 fragments eluted at 15 and 20 min derive from the variant V127 and L127. Interestingly, the observed retention times (t_R) of all triptic fragments significantly correlated ($r^2=0.91$) with the predicted values computed on the basis of the Rekker's constants as elaborated by Sasagawa et al. [42].

Deamidation is a hydrolytic reaction that depends only on protein sequence and conformation. There has been evolutionary selection in favour of large, functionally inert residues adjacent to N and Q, and some selection against polar residues, particularly H adjacent to N, and against D adjacent to Q [41]. N and Q occur most frequently on the surface of proteins and deamidation of Q is intrinsically slower than that of N in peptides and proteins. Deamidation of N makes the protein entourage more exposed, hydrophilic, and flexible and is one of the principal

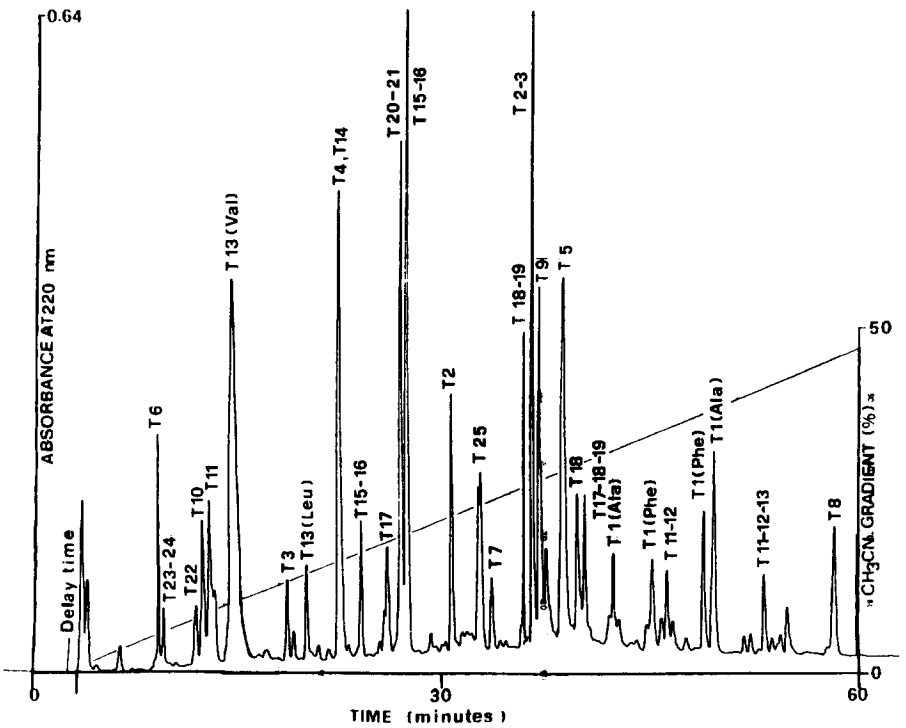

Fig. 6. Reversed-phase HPLC elution profile of a tryptic digest of 50 nmol of bGH. Digestion with trypsin of the reduced and carboxymethylated bGH (enzyme/protein ratio of 1:100) was allowed to proceed for 6 h at 37°C. Digest was chromatographed on an Aquapore RP-300 column (Brownlee Labs., Santa Clara, CA) at 23°C with an HPLC pump mod. Familic-300-S, Uvidec-100-V spectrophotometer and GP-A40 gradient programmer (Japan Spectroscopic Co., Tokyo, Japan). Peptides were eluted with a 60-min linear gradient from 0.1% aqueous TFA to 50% mixture with 0.075% TFA in acetonitrile, at a flow-rate of 2 ml/min. Eluting peptides were detected by their absorbance at 220 nm. Reprinted from Ref. [41] with permission.

causes of irreversible denaturation of proteins. These findings have important implications for the role of N and Q in protein folding, assembly into biologically active complexes and breakdown [43].

Phosphorylated molecules of GH have been demonstrated in sheep whose GH has a 98% homology with bGH, but not in bovines. Rat and chicken also have phosphorylated molecules.

Isoaspartate derivatization seems to occur as a function of in vitro aging. In recombinant bGH, the sites involved are N99 and D128, but in the pituitary hormone this post-translational modification can only occur at D128 [44].

Partial denaturation of bGH results in conformational perturbation that causes it to aggregate and form an associated intermediate [38]. Sequence 110–127 is probably amphipathic and critical to this aggregation. The hydrophobic surface of this amphipathic region was expanded by replacing K112 with L, thus increasing the intermolecular association of the partially denatured species [38], that might increase the apparent hormonal heterogeneity.

2.2. Three-dimensional structure of bGH

Procedures for the crystallisation of bGH have been reported since 1948 [45,46] but these procedures produced a very small crystal, not suitable for crystallographic studies. Efforts to obtain more suitable crystals were hampered for years by the heterogeneity of this hormone isolated from natural sources. Today's purification procedures for bGH yield a highly purified protein, but its three-dimensional structure has still not been described, and we

still have to refer to the crystal structure models of other species GH, such as the porcine GH (pGH) [20] and the human GH (hGH) [47].

Since there is a high degree of sequence identity between GH from bovine and porcine species (only 18 residues well scattered within the molecule out of 191 are different), the bGH three-dimensional structure is likely is be similar. In brief, the pGH structure consists of four antiparallel α-helices arranged in a left twisted helical bundle, the first two helices running 'up–up' in parallel fashion and the other two running 'down–down'. This peculiar structure is very similar to that of hGH and seems to be a conserved biological feature among members of the hematopoietic super-family [4].

Recently, the arrangement of human GH (hGH) in the crystal structure of the complex with its receptor has also been elucidated [47]. The receptor is a dimeric protein that binds one molecule of GH in two distinct regions; the two sites on the hormone are named sites I and II.

The structural homology between the GH (hGH and bGH show 66% of sequence identity) and their receptors, and the cross-reactivity at the receptor [48] are in favour of a similarity between the hGH–receptor and bGH–receptor complexes.

Like hGH, the bGH residues involved in the binding with its receptor should be largely discontinuous and located on both helix and loop–coil regions on opposite sides of the four-helix bundle. By aligning hGH and bGH sequences to maximise homologies, some deductions can be made regarding the amino acid residues of bGH involved in receptor binding (Fig. 1). Site I might involve residues on the C-terminal portion of helix-4 (residues 164–191), some residues on helix-1 (residues 20–26), and on the loop between helix-1 and helix 2 (residues 42–46 and 61–66). Site II could include residues of the N-terminal portion of helix-1 (residues 3–19) and a few residues of helix-3.

Exceptions in the topography of hGH which were not described for pGH (and thus probably also absent in bGH) are the two short helices in the connecting segments between helices 1 and 2 (residues in bGH could be 39–47 and 64–70) and a third between helices 2 and 3 (residues in bGH could be 95–100). Since the first two are involved in contacts between hormone and receptor, they may represent conformational changes in the hormone on receptor binding [4].

According to Wells and De Vos [4], the aminoacyl residues with a significant contact area with the receptor can be defined as structural epitopes, whereas residues that show reduced affinity binding when changed with an alanine are defined as functional epitopes. The X-ray structure of the hGH-receptor complex together with homologue and alanine scanning of the binding sites on hGH served to locate precisely some of the hGH residues involved in structural and functional epitope contact with its receptor. On the basis of the sequence and structural similarities between hGH and bGH, it can be concluded that for bGH the key residues for the functional epitope in site I are F11, F54, E56, I 58, T62, K64, E66, Q68, S163, K171, R177, V178, C181, G185 and F2, M5, G9, D115, E117 in site II. Likewise, crucial residues for the structural epitope of bGH site I could include a few residues of helices 1 and 4 (H20, H22, R166, K167, E173) and residues N13, R17, Q19 or H20, R108 for site II.

3. Topographic analysis of the antigenic epitopes of bGH

Antigenic determinants on hormone molecules can be sequential (or continuous), represented by peptides of defined sequence, or conformational (or discontinuous), requiring close proximity of several different sequence regions on the molecule. The antigenic determinants of the bGH molecule have been intensively investigated by a variety of techniques, and both sequential and conformational epitopes have been reported [33,34,48–59]. In general, four to five major antigenic sites have been described on the bGH molecule, the majority apparently involving the 15 kDa amino terminal of the hormone.

Recently, two different techniques have provided precise information on the location of some continuous epitopes recognised by polyclonal anti-recombinant bGH antisera: (1) Beattie et al. [50,51] mapped the continuous epitopes recognised by rabbit, guinea pig and mouse anti-bGH antisera by the method referred to as 'multiple-pin epitope scanning'. The technique, developed by Geysen et al. in 1984 [52],

is based on assays of the antigenicity of all possible overlapping octapeptides covering the full amino acid sequence of an immunologically important protein. Since antibodies produced against the native protein are, by definition, directed to antigenic epitopes, it follows that peptides reacting with these antibodies must contain elements of the epitope; (2) Aston et al. [53] raised polyclonal anti-sera in sheep to peptide fragments of bGH, and examined their reactivity toward the intact protein in solid- and solution-phase assays. Peptide that gave rise to antibodies reacting with the intact protein should mimic an epitope on the protein surface.

The continuous epitopes identified by these methods on the bGH molecule lie in the following amino acid sequences (in brackets epitopes defined by Aston et al. [53]): 24–43, (32–46), (53–73), 101–110, 130–152, (134–154), (120–140), 179–191 and (167–191).

The sequence between 130–154 seems to be a major antigenic site. It is clearly recognised by all the antisera tested, independently of the host species. Fig. 7 reportes examples when mouse, guinea pig and rabbit polyclonal anti-rbGH antisera were epitope-scanned using an array of immobilised octapeptides representing the entire GH molecule. A common feature of the profile is the reactivity of each antiserum toward the 139–152 region.

Major epitopes are generally believed to be found in regions of non-conserved sequence; however, comparison of mouse, rabbit, ovine and bovine GH sequences – unfortunately the guinea pig GH primary structure is not yet known – within residues 130–154, indicates a high degree of conservation: 23/25 (92%) for sheep, 20/25 (80%) for rabbit and 21/25 (84%) for mouse. The immunological properties of the 130–154 immunodominant region are probably conferred by its surface accessibility in the three-dimensional structure of the hormone. It should be recalled that the Q140 and N148 residues are known to be subject to deamidation. This renders this portion of the molecule more hydrophilic and might enhance its surface accessibility and immunogenicity [41].

Bonford and Aston [54] illustrated the greater dependence of GH immunogenicity on folding and structure than on degree of sequence homology of the peptide antigen, using oGH-peptide fragments to induce autoimmunisation of sheep. Despite the fact that the synthetic peptides used for vaccination and the corresponding sequence of the recipient animal GH were identical, there was good autoimmunisation after three injections of the antigen.

These and other data (i.e. EIA methods that can properly distinguish between bGH and oGH) underline the close species-specific antigenic structure of GH. On the other hand, anti-bGH MAbs have been seen to cross-react with GH from other species, including hGH, and with bovine PRL (bPRL). For example, Krivi and Rowold [33] showed that 4 out of 29 (4/29) anti-bGH MAb bound to pGH, and 5/29 reacted with hGH as well as bGH. Retegui and Paladini [57] found that 5/5 anti-bGH MAb bound oGH, 5/5 eGH, and 4/5 pGH. Secchi et al. [34] reported that, with different degrees of cross-reactivity, 7/7 anti-bGH MAb also recognised oGH, 6/7 cGH, 6/7 pGH, 5/7 rGH, 2/7 hGH and 1/7 bPRL. Some of the cross-reacting anti-bGH–MAb described by Retegui and Paladini [57] and Secchi et al. [34] showed heteroclitic behaviour, reacting with more affinity with heterologous GH.

Beattie and Holder [49] provided a convincing explanation for this pattern of MAb cross-reacting with heterologous antigen. They justified the ability of a MAb to cross-react in solution with oGH, bGH, pGH and rGH by demonstrating that it recognises a sequential epitope conserved among these species. In fact, the sequence 94–100 (SRVFTNS), that is the minimum unit to retain full recognition of the MAb, is identical in bGH, oGH and pGH. In rGH residue V96 is replaced by I but replacement analysis showed that this substitution does not inhibit binding of the MAb to the heptapeptide.

In general terms, inspection of the secondary and tertiary structure indicates that all continuous epitopes identified until now tend to lie in regions of the protein which are less well ordered (i.e. loop/coil type structure). For example, the most immunogenic region of bGH is part of the loop/coil structure 130–152 connecting helices 3 and 4 in the native GH molecule. However, this observation may relate not to any innate property of the immune response to bGH, but rather to the inability of the helix-derived peptides to adopt the appropriate conformation, precluding any recognition by antibodies raised to this region on the native protein molecule.

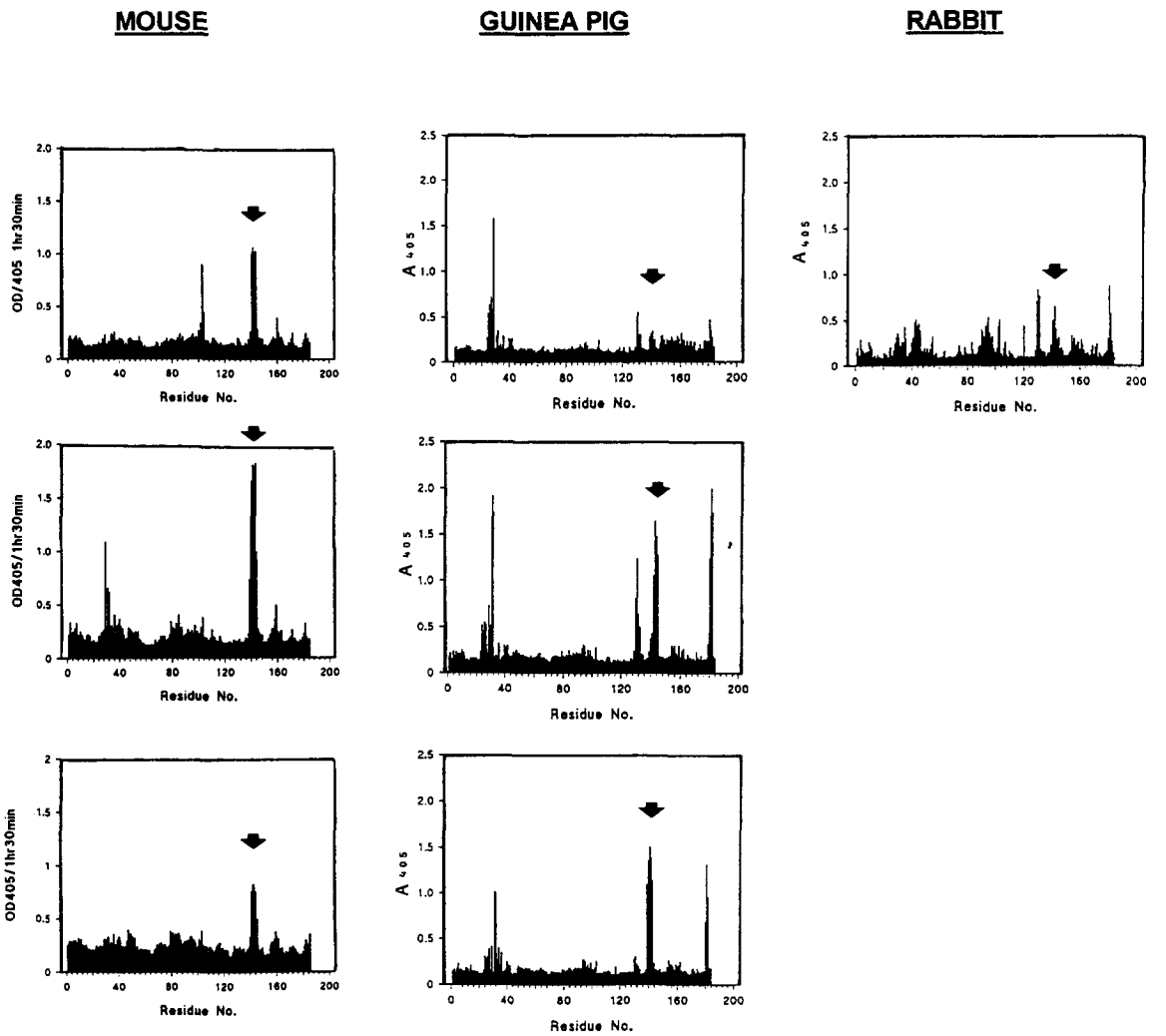

Fig. 7. Multipin peptide analysis of the continous epitopes recognised by three mouse, three guinea pig and one rabbit polyclonal anti-rbGH sera. Octamers representing the entire sequence of bGH were assembled with a one-residue overlap onto activated polyethylene pins. The 184 peptides were accommodated on blocks of 96 pins. Pins were then placed in a solution of the first antibody (i.e. mouse or guinea pig or rabbit serum) and incubated overnight at 4°C. After washing, blocks of pins were incubated with appropriate second-antibody–peroxidase conjugate. Pin blocks were washed as before and bound antibody was detected by immersion of pins in a buffer containing 0.05% 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) and 0.01% hydrogen peroxide. Colour development was monitored over a period of 30–120 min and absorbance was determined at 405 nm. The abscissa indicates the position within the GH molecule of the N-terminal residue for each pin-bound octapeptide, e.g. number 40 on the *x*-axis indicates octapeptide 40–47. Black arrows mark the epitope between residues 130–154. Adapted from Ref. [50,51] with permission.

In a wider sense, studies of the functional dissection of the bGH molecule into separate active domains may help clarify the elucidation of the structural features responsible for its activities. One example is the detailed description of the sequences on the bGH molecule recognised by antibodies that enhance the growth response of dwarf mice injected with bGH.

3.1. MAb-mediated enhancement of bGH biological activity

Enhancement of hormonal activity by MAb or polyclonal antisera has been observed in a number of systems both in vitro and in vivo, and the antibody-mediated enhancement of the growth promoting activity of both primate and non-primate GH is well established in vivo [52]. Enhancement of hormonal activity by specific antisera or MAb is clearly epitope-related, depending on either linear sequences or discontinuous regions of the hormone.

The use of anti-peptide antisera cross-reacting with the native hormone and the multiple-pin epitope scanning technique led to the identification of sequence regions on bGH associated with enhancement [49,52]. The growth rate was determined in the dwarf mouse in vivo by bioassay, by the incorporation of [^{35}S]sulfate into rib cartilage. Antibodies specifically directed to sequence regions (in brackets the sequence reported by Beattie and Holder [49]) 35–53, (91–102), 120–140 and 134–154 significantly and reproducibly enhanced the somatogenic activity of the hormone on growth rate. Taking due care in extrapolating from primate to non-primate GH structures, it can be noted that all the enhancing MAb recognise sites distant from areas involved in receptor binding; only the hGH sequence 35–53 is involved in receptor binding; however, as discussed in Section 2.2, in the bGH molecule this area presumably has a different structure. Thus, it seems that the binding of the enhancing antibody to the hormone should not be incompatible with subsequent binding of the complex to the receptor. However, several authors reported that enhancing MAb in vitro can either inhibit or improve GH binding to its receptor on cell surface, and there was no clear correlation between the effects of MAb on hormone receptor binding in vitro and the growth enhancement phenomenon in vivo [60].

Although the molecular mechanisms underlying MAb-mediated enhancement of GH biological activity by selected polyclonal and monoclonal antibodies are still unclear, these findings suggested that a 'restriction' mechanism operates in vivo. MAb enhancing growth permit GH binding only to receptors involved in the growth process. If topographically distinct 'active regions' exist on the bGH molecule, a site-directed antiserum might inhibit one activity but not another. The fact that several GH receptors present structural and presumably functional heterogeneity supports this.

Many other possible mechanisms have been suggested to explain MAb-mediated enhancement of biological hormonal activity. These include slow release of the hormone from the antibody, resulting in prolongation of its systemic half-life or reduced GH degradation in tissues; MAb-induced allosteric modification of GH receptor recognition sites (this could influence the hormone's receptor affinity or the time of receptor occupancy, prolonging receptor activation and amplifying intracellular events); Fc-region mediated targeting and bivalency of the MAb (though in the case of GH enhancement, univalent Fab′ Ab fragments are also effective, excluding bivalency of Fc-mediated targeting as the basis of the effect). These and other possible mechanisms have been reviewed in more detail by Aston et al. [61].

Identification of the antigenic peptide sequence from oGH which elicits enhancing antisera in ewes [53], and the significant increases in lactation of ewes treated with GH complexed with growth-promoting MAb [62], together with the potentiation of endogenous GH activity by administration of particular MAb to the marmoset [63], may provide the basis for influencing animal production by growth-promoting vaccine that immunologically regulates endogenous hormonal activity.

4. Detection of bGH

A protein can be detected in a biological sample by measuring its biological activity. However, the complexity of available methods for testing GH bioactivity has led to the development of alternative methods, mostly based on the hormones' immunological properties, which are strictly species-specific.

To develop an immunochemical assay of GH it is necessary to bear in mind that several forms of the hormone coexist in the plasma, with the M_r 22 000 form dominating. In addition, some immunoassays may be influenced by the presence of circulating GH-binding proteins [2]. This could be the case if

antibodies with low affinity for GH are employed, or if the assay uses MAb whose GH epitope is masked by the hormone binding with its binding protein. Also the presence in plasma of homologous hormones, such as prolactin and placental lactogen, can give rise to erroneous results.

In view of the economic importance of the bovine species, various immunoassays have been developed since 1969 to measure bGH concentrations in plasma and biological fluids [64]. Initial methods were isotopic [64–66] and sensitivity was about 2.5 ng/ml [66,67]. Such RIA have become increasingly regarded as undesirable in the last few years because they use radioactive materials. ELISA methods are safer, require no expensive instruments and overcome the problem of disposing of radioactive wastes. In addition, the development of amplification systems enabled us to reach sensitivity of the order of pg of hormone/ml [68]. This extreme sensitivity is a must when the sample is urine, as only 0.01% of the amount of GH produced is excreted in the urine and GH concentrations in urine are several hundred times lower than in plasma [68].

A rapid and sensitive 'sandwich-type' ELISA was developed in 1988 by Secchi et al. [69]. The method exploits the amplification of the biotin–avidin interaction and detects levels of bGH as low as 0.1 ng/ml. The test is specific enough to discriminate between bGH and GH from different species, including ovine GH, whose amino acid sequence differs only at three positions (cross-reactivity 73.7%). The method also distinguishes between pituitary bGH and a recombinant bGH (from Eli Lilly) differing in a highly hydrophilic octapeptide added to the N-terminus of the molecule (cross-reactivity 77.2%) [55]. Fig. 8 shows the curves obtained with different concentrations of purified ovine, canine, porcine, rat and human GH, bPRL, and recombinant bGH in the ELISA specific for bGH. The same quantitative immunoassay was used to study bGH plasma patterns of animals in physiological conditions and after pharmacological treatments [70–72]. The episodic pattern of GH secretion of all mammalian species tested so far makes it unreliable to use only one blood sample as a basis for establishing the plasma GH concentration. Researchers have resorted to continuous sampling or intermittent sampling at frequent intervals to estimate GH concentrations,

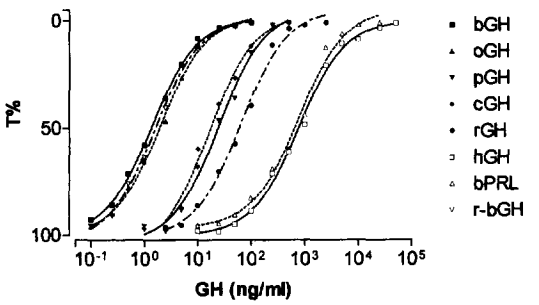

Fig. 8. Cross reactivity of different concentrations of purified ovine (oGH), canine (cGH), porcine (pGH), rat (rGH), human (hGH) and recombinant bovine GH (r-bGH) and bovine PRL (bPRL) assayed by an ELISA specific for pituitary bGH [65]. Polystyrene microtiter plates were coated with affinity purified anti-bGH antibodies. Remaining reactive sites on the plastic were saturated with BSA, and, after washing, serial dilutions of antigens were added to the plates. After incubation at 4°C for 16 h biotinylated anti-bGH antibody was added. Bound antibodies were detected using peroxidase-conjugated avidin. Enzymatic reaction was carried out by adding a solution containing 3 n*M* *o*-phenylenediamine dihydrochloride and 0.01% hydrogen peroxide. The reaction was stopped by addition of H_2SO_4 and the absorbance was read at 492 nm. Curves are plotted as transmittance (*T*%) vs. log of ng/ml of GH.

then analysing the patterns by suitable algorithms. The results of one such study in bovine is shown in Fig. 9.

The use of monoclonal antibodies directed against specific epitopes of the bGH could increase the specificity of the ELISA. The most striking examples of fine specificity of antibodies are MAb that distinguish between pituitary hGH and recombinant methionyl-hGH, which has an extra methionine at the amino terminal of the molecule [73].

Unfortunately, anti-bGH MAb are often of low affinity, because of the low immunogenicity of the hormone [34], leading to scant sensitivity of the immunoassays. Results are better using combinations of mono- and polyclonal antibodies in 'sandwich-type' assays [55,74], with MAb as first antibody and polyclonal as second labeled antibodies.

Alternative methods for quantitative determination of GH are immunoblotting or affinity chromatography [75,76]. The immunoblotting assay [75] is relatively easy, and its sensitivity is in the nanogram range. However, it requires electrophoretic and densitometric instruments, and only 10–20 samples per

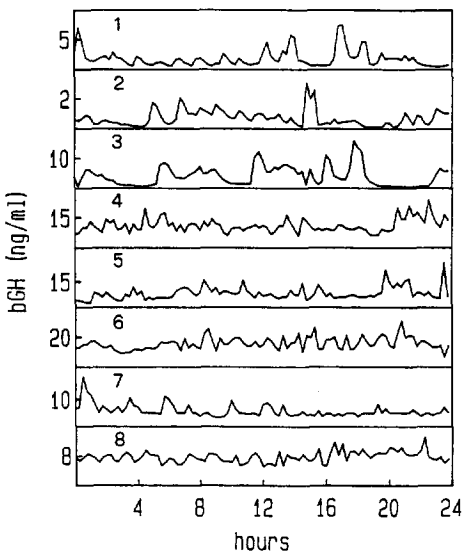

Fig. 9. Circadian plasma secretory patterns of bGH in eight 16-month-old Friesian heifers, obtained by 15-min interval sampling. Reprinted from Ref. [71] with permission.

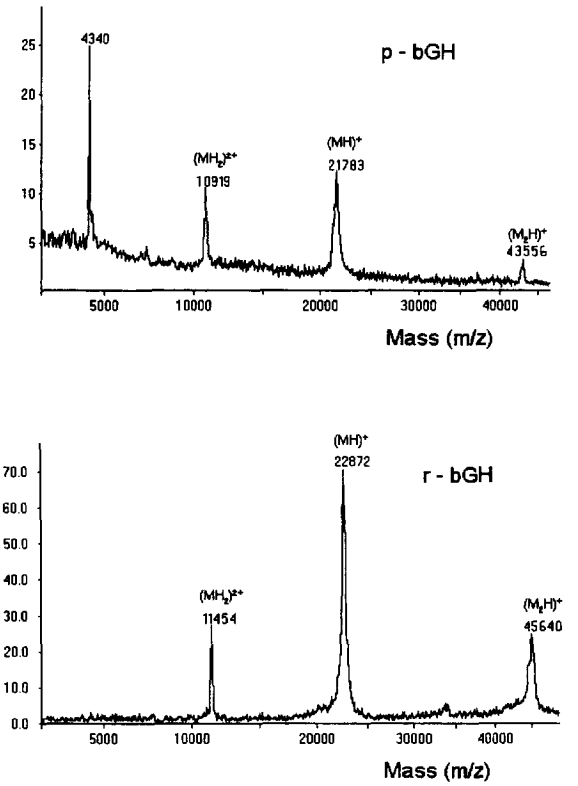

Fig. 10. LD–MS spectra of the p-bGH and r-bGH. M_r values were obtained using a Laser Mat (Finningan Mat, Hemel Hempstead, UK). Light from a pulsed nitrogen laser (337 nm) was focused onto the sample target at a power density around 106 W/cm^2. The ions desorbed by each laser pulse were accelerated to 20 keV energy along a 0.5-m time-of-flight (TOF) drift tube. Typically, the spectra were taken by adding together ten single-shot spectra at a laser flunk just above the threshold for ion production. Calibration was performed with a low M_r oligosaccaride dextran. The m/z values of the samples were calculated from this curve by an external standard calibration method. Presence of either multiple ionisation or aggregate forms are indicated as follows: $(MH)^+$=monomer, single ionisation; $(M_2H)^+$=dimer, single ionisation; $(MH_2)^{2+}$=monomer, double ionisation. Reprinted from Ref. [77] with permission.

run can be quantified. The affinity chromatography method [76], developed for hGH, is highly sensitive (range 0.002 pg to 2 ng of hormone, which is 100–1000 times better than the classical sandwich assays). In view of its complexity, the assay needs to be adapted for automation, but no applications of the method to routine determinations have yet been described.

The most recent method for detection of bGH employs matrix-assisted laser desorption mass spectrometry (LD–MS). LD–MS rapidly and accurately measures the molecular mass (M_r) of intact proteins, and the method is sensitive enough for the characterisation of protein variants and modifications. Thus, any post-translational modification or amino acid substitution resulting in a difference of a single mass unit can be detected. The method has been employed to detect recombinant bGH in sustained-release preparations, but could also be applied to plasma samples [77]. Fig. 10 shows the LD–MS spectra of the p-bGH and r-bGH (NH_2–M–F–P–L–D–D–D–D–K–F from Eli Lilly) used as standards. Besides the characteristic single protonated protein ion (MH^+) (M_r 21 783 for the p-bGH and M_r 22 872 for the r-bGH) and the double protonated ion $(MH_2)^{2+}$ (M_r 10 919 and M_r 11 454, respectively), both spectra showed peaks corresponding to polymeric forms. The M_r of the p- and r-bGH acquired differed by less than 0.3% from the hormonal M_r values calculated from the sequences (21 819 for the p-bGH and 22 825 for the r-bGH).

The LD–MS technique, coupled with affinity purification of the sample, has been used for quantitative detection of miotoxins present in the blood,

with an absolute detection limit between 50 and 5 fmol [78]. A similar mass spectrometry immunoassay could be developed for other antigens, including GH.

5. bGH gene

The GH gene belongs to a multi-gene family whose members are thought to be derived from duplication of a single ancestral gene that gave rise to the separate PRL and GH lineage [1,2]. Nucleotide sequences are available for ten mammalian coding sequences, including those derived from human, rat, mouse, hamster, mink, horse (incomplete sequence), sheep, goat and bovine [3]. The bGH gene contains approximately 1793 nucleotides, consists of four introns (A–D) and five exons (1–5), and has been mapped to 19q chromosome at 1–7qter location [79]. Twenty-six amino acids encoded by exon I and part of exon II direct the synthesis of the bGH amino-terminal signal peptide, which is not retained in the mature protein and functions as a secretory traffic signal [37].

Analysis of total bovine DNA suggested there may be several slightly different (non-allelic) copies of the bGH gene in the bovine genome, but their functional significance remains to be explored [80]. In fact, while in humans highly homologous GH genes are expressed in distinct tissues (the hGH-N gene in the pituitary, the hGH-V and the chorionic somatomammotropin CS-A and CS-B genes in the placenta), in the bovine, like in all the other non-primate mammals, the bGH gene seems to be transcribed only in a restricted cell-specific pattern in a subset of anterior pituitary cells, known as the somatotrophs.

Transcription of the GH gene in mammals depends mainly on transduction to the somatotroph nucleus of the signals of two antagonistic hypothalamic neurohormones: somatostatin (SS) and GH-releasing hormone (GHRH). Recent studies of hGH gene expression [81,82], showed: (1) that members of the CREB/ATF-1 transcriptional factor family are the final communication link between cell surface and genome and (2) that the functional transcriptional unit on the hGH gene promoter comprises two CREB/ATF-1 binding motifs and a GHF-1 binding site. Fig. 11 provides a schematic model of hGH gene expression in somatotrophs. After binding to specific seven-helix motif transmembrane receptors GH–RH stimulates and SS inhibits intracellular adenylate cyclase. The increase of cyclic AMP (cAMP) leads to activation of a cAMP-dependent protein kinase A (PKA), and to the subsequent dissociation of its catalytic (C) and regulatory (R) subunits. The C subunit, after translocation to the nucleus, binds to consensus sites (RRPSY or A-kinase Box) and phosphorylates serine residues of protein members of the leucin zipper (b-ZIP) family, labelled CREB/ATF-1 transcriptional factors. Phosphorilation of CREB/ATF-1 by PKA promotes dimerization and increases its efficacy as a transcriptional activator. Once dimerizated, CREB/ATF-1 binds to TGACGTCA sequences on the gene promoter regions, known as cAMP-responsive elements (CRE). Protein binding to the CRE is a signal for activation of gene transduction. In this context whether phosphorylation and dimerization by PKA influences the DNA-binding of CREB continues to be controversial [83].

Two partial CRE sequences (CGTCA) have been located on the hGH promoter (pCRE between −99/−95 and dCRE −187/−183 bp upstream of the transcriptional start site), and directly regulate hGH gene transcription. Canonical CRE sequences lie on the gene promoter of another gene transcriptional factor, the GHF-1. Transcription of the GHF-1 gene produces a protein belonging to the Pit–Oct–Unc (POU) domain family, that is the most important cell-specific activator of GH gene expression (the GHF-1 binding sites are at positions −123/−112 and −65/−92 of the hGH promoter).

In addition to the previously described sequences of the hGH promoter which mediate specific hormonal transcriptional regulation, binding sites for more ubiquitous transcriptional factors have been described. Sp1 binds to the region between nucleotides −115 to −139, partially overlapping the distal GHF-1 binding site. A binding site for the upstream stimulatory factor (USF) is present between nucleotides −253 to −266. The sequence from position −267 to −289 binds nuclear factor-I (NF-1), and activator protein-2 (AP-2) is known to bind to the same region in a mutually exclusive manner [84].

The exact mechanism regulating transcription of

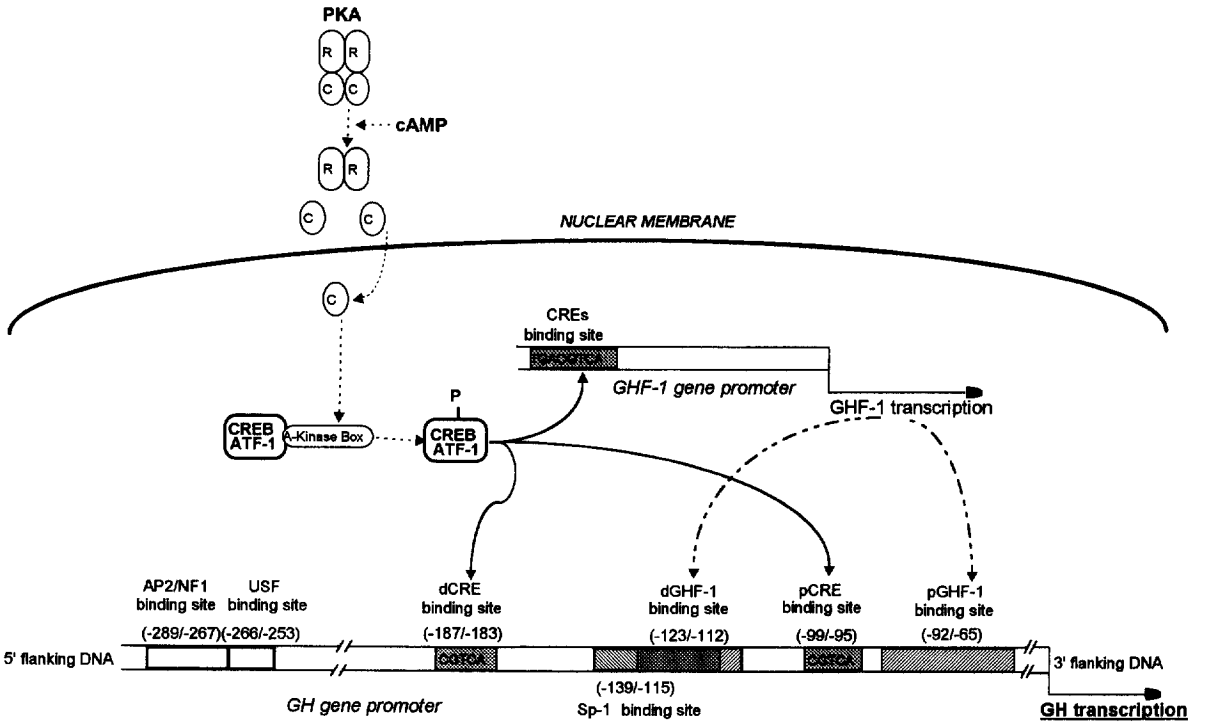

Fig. 11. Schematic diagram representing possible pathways of GH gene expression in somatotrophs. Intracellular cAMP stimulates cAMP-dependent protein kinase A (PKA), leading to dissociation of its catalytic (C) subunits from regulatory (R) subunits. The catalytic subunit after translocation to the nucleus binds to a specific A-kinase box (RRPSY) and phosphorylates transcription factors of the CREB/ATF-1 family (cAMP-responsive element binding protein), which in turn activates transcription by binding to cAMP-responsive elements (CRE). Two partial CRE on the GH promoter and a canonical CRE sequence on the GHF-1 gene promoter are shown. The transcription factor GHF-1 will in turn activate expression of the GH gene. Binding sites for ubiquitous transcriptional factors are also shown: upstream stimulatory factor (USF), nuclear factor-I (NF-I), activator protein-2 (AP-2) and Sp1.

the GH gene in the bovine species is still unclear, but its regulation is probably well conserved through vertebrate evolution. Two out of three DNA sequences crucial for the regulation of hGH gene expression lie within a 40 bp region (positions −137 to −98 of the hGH gene promoter) with a very high degree of sequence similarity among mammals, and sequences homologous to the mammalian Pit-1/GHF-1 binding site are also present in the rainbow trout gene promoter [81].

A recent study [85] showed that the expression of bGH gene is regulated negatively by three sequences, named negative regulatory elements (NRE), located at the 5′-flanking region between nucleotides −315 and −306, −269 and −240, and nucleotides −146 and −110. The presence of negative regulatory sites in the promoter region of GH gene has been previously described in human and rat. The NRE identified on the bovine GH gene have sequence similar to each other, and to several DNA elements which are known to bind YY1. This is a multifunctional transcription factor (known also as NF-1, delta, or UCRBP) that acts as a negative regulator for several genes in human, rat as well as in adeno-associated viruses.

Bovine GH-RNA post-transcriptional events have now been studied and bGH offers a new paradigm for efficient mRNA polyadenylation in the absence of typical polyadenylation sequences (the required sequences are present as a dispersed rather than discrete element) [86], and is a model system for studying how to achieve intron retention and how intron retention-containing m-RNA are allowed into the cytoplasm [87]. Retention of the last intron

(intron D) in bGH primary-mRNA and its transport to the cytoplasm depends on the balance between two splice sites and a third, downstream purine-rich exonic splicing enhancer (ESE) which, as usual, is not part of a very small sequence, but seems to be composed of multiple sequences spread throughout a 115bp-nucleotide region of exon 5.

Translation of the intron D containing bGH-mRNA generates a bGH-related isoform with a 125-amino acid N-terminus identical to wild type GH, followed by an altered C-terminus of 108 AA. The corresponding protein has not been found in the pituitary and the role of this bGH isoform is not yet known [88]. However, identical alternative splicing of the hGH gene pre-mRNA expressed in the placenta (hGH-V2) suggests it has physiological importance. The corresponding protein to hGH-V2 has been found as a membrane-associated non-secretory 26 kDa protein, with a carboxyl-terminus tail that is completely different from other hGH isohormones.

Acknowledgments

The present paper is dedicated to the memory of E. Martini, Professor of Physiology and Biological Chemistry at the University of Milan. The authors thank Anna Berrini for skilled technical assistance in experimental parts performed. Part of the work was funded by M.U.R.S.T. (60 and 40% Progetto Biochimica di Sistemi Sopramolecolari; funds to C.S.).

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