Purification and biochemical characterization of recombinant human placental growth hormone produced in *Escherichia coli*

Ahmed IGOUT,*†, Jozef VAN BEEUMEN,†, Francis FRANKENNE,* Marie-Louise SCIPPO,* Bart DEVREESE† and Georges HENNEN*

*Biochimie et Laboratoire d'Endocrinologie, Tour de Pathologie, B23-4000-Liege, Belgium

and †Vakgroep Biochimie, Fysiologie en Microbiologie, K.L. Ledganckstraat 35, 9000 Gent, Belgium

The *hGH-V* (or *hGH-2*) gene codes for human placental growth hormone (hPGH). Secretion of hPGH is continuous, in contrast with the pulsed secretion of pituitary growth hormone (hGH) which it progressively replaces in the maternal bloodstream. *hGH-V* cDNA has previously been cloned and isolated. Analysis of its nucleotide sequence has revealed a 191-residue protein, hPGH, differing from hGH at 13 positions. The calculated pI is more basic than that of the pituitary hormone. Here we have inserted *hGH-V* cDNA into the pIN-III-ompA3 plasmid in order to produce hPGH in its native form in *Escherichia coli* D1210. Expression of *hGH-V* cDNA in *E. coli* is significantly lower than

that of *hGH* cDNA with the same expression system. The hPGH produced in *E. coli* was purified in quantities sufficient to allow its biochemical and immunochemical characterization. The molecular mass of the protein was determined by electrospray m.s. The determined mass, 22 320 Da, agrees well with the molecular mass calculated from the translated cDNA sequence, assuming the presence of two disulphide bridges. Having established the technique for producing hPGH with a primary structure identical to the natural, non-glycosylated, 22 kDa isoform, we can now plan the full physicochemical and pharmaceutical characterization of this new hormonal entity.

INTRODUCTION

The *hGH-V* gene belongs to the *GH* gene family. These genes are located on chromosome 17 where they appear in the following order, from 5' to 3': *GH-N*, *CS-L*, *CS-A*, *GH-V*, *CS-B* (Owerbach et al., 1980; Barsh et al., 1983; Hirt et al., 1987; Chen et al., 1989). The *hGH-N* gene is expressed in the pituitary gland and codes for human pituitary growth hormone (hGH). As a secreted product of the anterior pituitary, hGH promotes growth and development and is involved in regulating a variety of anabolic processes (Bolander, 1989).

Pathological absence of hGH induces dwarfism or reduces statural growth, situations which can be corrected by administering exogenous hormone. During human pregnancy, production of maternal pituitary GH is progressively suppressed while placental GH (hPGH) is synthesized in the placenta (Hennen et al., 1985a,b, 1986; Frankenne et al., 1988) and secreted continuously and in increasing amounts into the maternal bloodstream (Erikson et al., 1989). hPGH contains the same total number of amino acids as hGH but differs at 13 positions and, moreover, exists in a minor, glycosylated form (Frankenne et al., 1990). The *hGH-V* gene, which is expressed in the placenta, yields an 800-nucleotide mRNA (Igout et al., 1988) and a 1250-nucleotide mRNA resulting from alternative splicing (Cooke et al., 1988). The 800-nucleotide mRNA codes for the 22 kDa hPGH. Specific expression of the *hGH-V* gene in the placenta has been demonstrated by *in situ* hybridization (Scippo et al., 1993) and by immunohistochemical localization (Jara et al., 1989). The *hGH-V* cDNA coding for the major 22 kDa form has been isolated (Igout et al., 1988).

hPGH has been purified from full-term placenta, but in amounts too low for extensive biochemical characterization. In order to obtain larger quantities of hPGH uncontaminated by other placental proteins, we have expressed its cDNA in *E. coli*. In this paper we report, for the first time, on the expression,

secretion, purification, and biochemical characterization of recombinant hPGH (rec-hPGH). To obtain a rec-hPGH similar to the natural, non-glycosylated 22 kDa isoform, we have cloned *hGH-V* cDNA into the pIN-III-ompA3 secretion vector (Ghrayeb et al., 1984).

EXPERIMENTAL

Materials

E. coli strain HB101 (Boyer and Rouland-Dussoix, 1969; Bolivar and Backman, 1979) was used as the bacterial host for cloning. Strain D1210 (Salder et al., 1980) was used for *hGH-V* cDNA expression. Plasmid pIN-III-ompA3 was a gift from M. J. Inoye (Stony Brook, NY, U.S.A.). The enzymes used in this study were purchased from Pharmacia and Boehringer-Mannheim (Belgium). Yeast extract, Bactotrypton-140, isopropyl- β -D-thiogalactopyranoside (IPTG) and phenylmethanesulphonyl fluoride (PMSF) were purchased from Pharmacia. The hGH used as a standard in the radioimmunoassay was MRC hGH 66/217 (London, U.K.). Two anti-GH monoclonal antibodies (mAb) named 5B4 and K24 (Gomez et al., 1984) were used to characterize rec-hPGH. All other reagents were commercial preparations of analytical grade or of the highest purity available.

A home-made 50-litre bioreactor was used for preparative expression and a Multigen convertible bench-top culture apparatus F1000 for optimizing expression.

Culture, induction and release of the periplasmic proteins

A freshly streaked colony was used to inoculate a pre-culture grown overnight in L-broth medium with 100 μ g/ml ampicillin. An aliquot (2.5 ml) of this pre-culture was used to inoculate 30 litres of L-broth medium containing 150 μ g/ml ampicillin, or 1 litre of L-broth medium in a Multigen culture apparatus at a controlled temperature and oxygen delivery. When the absor-

Abbreviations used: hPGH, human placental growth hormone; hGH, human pituitary growth hormone; rec-hPGH, recombinant hPGH; IPTG, isopropyl- β -D-thiogalactopyranoside; PMSF, phenylmethanesulphonyl fluoride; mAb, monoclonal antibody.

† To whom correspondence should be addressed.

bance at 600 nm reached 0.75, expression of the chimeric gene *ompA-hGH-V* was induced with 50 μ M IPTG. The induction temperature was 30 or 37 °C; aliquots (100 ml) were taken to monitor the time course of induction at each temperature. To produce large quantities of rec-hPGH, 30-litre batches of L-broth medium were inoculated with 5 ml of overnight pre-culture. At an absorbance (600 nm) of 0.75, the temperature was reduced from 37 °C to 30 °C. When the absorbance value reached 0.8 (600 nm), expression was induced with 50 μ M IPTG. Proteins were released from the periplasm 9 h later by treating with lysozyme and EDTA to form spheroplasts as previously described by Koshland and Botstein (1980) and Osborn et al. (1972). Cells were harvested at 4 °C by centrifuging for 15 min at 4000 *g*. All subsequent steps were carried out at 4 °C. Pellets corresponding to 7.5 litres of cell culture were suspended in 400 ml of a solution containing 0.1 M Tris/HCl (pH 7.2), 0.5 M sucrose and 0.5 mM EDTA, immediately followed by 40 ml of lysozyme (2 mg/ml) and 400 ml of cold distilled water. After addition of 1 ml of PMSF solution (1 mg/ml in ethanol), the suspension was incubated on ice and the efficiency of the spheroplasting procedure monitored by phase-contrast microscopy. Spheroplasts were stabilized by adding 22.5 ml of 1 M MgCl₂. The spheroplasts were pelleted by centrifugation for 15 min at 4 °C at 4000 *g*. The pellet was resuspended in water, sonicated, and saved as a membrane/cytoplasm fraction.

Purification and analysis of rec-hPGH

The supernatant from the spheroplast treatment, which contained the periplasmic proteins, was mixed for 12 h with (NH₄)₂SO₄ at 25% saturation and the precipitate removed by centrifugation for 2 h at 4500 *g*. The (NH₄)₂SO₄ concentration was brought to 60% saturation for 16 additional hours. The 25–60% (NH₄)₂SO₄ precipitate was dissolved in 60 ml of 50 mM NH₄HCO₃/NH₃, pH 9.5, containing 1.6 M urea. The eluate was applied to a column of fast-flow Q-Sepharose. Proteins were eluted by raising the NH₄HCO₃/NH₃ concentration in the eluent from 50 to 600 mM. Aliquots of different eluted fractions were concentrated and analysed by SDS/PAGE. Fractions containing rec-hPGH were pooled and lyophilized. To prepare homogeneous rec-hPGH, the lyophilized fraction was subjected to gel filtration on a Sephacryl S200 HR 16/100 column and eluted with 150 mM NH₄HCO₃/NH₃, pH 9.5.

SDS/PAGE analysis and Western blotting

Electrophoresis on 15% (w/v) polyacrylamide gels in the presence of SDS was performed according to the method of Laemmli (1970). The gels were stained with Coomassie Blue. Western blotting was performed according to Towbin et al. (1979). Incubation with mAbs, and with antiserum, and staining were performed according to Frankenke et al. (1988).

Isoelectrofocusing

A fraction enriched in rec-hPGH was applied to Immobiline (LKB) in the 7–10 pH range and focused according to Bjellquist and Ek (1982). The gel was then cut into 0.5-cm fragments which were homogenized and eluted in 2 ml of distilled water. The pH was measured in each eluate. After centrifugation at 4500 *g* for 30 min, rec-hPGH was assayed in each fraction.

Amino-acid-sequence determination and computer analysis

Rec-hPGH sequencing was carried out with a 477A pulsed-liquid sequencer according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, U.S.A.).

The theoretical isoelectric point was calculated with the PC GENE program. Prediction of the prokaryotic secretory signal sequence of the *ompA/hPGH* hybrid was done according to von Heijne (1986). The hydropathy profile was determined by the method of Kyte and Doolittle (1982).

M.s. analysis

The molecular mass of rec-hPGH was determined with a BIO-Q triple quad electrospray-ionization mass spectrometer (Fisons Instruments, Altrincham, Cheshire, U.K.). Sample (100 pmol) was dissolved in 10 μ l of methanol/water/acetic acid (99:99:2, by vol.), then injected into the electrospray with a Rheodyne 5717 injector. The sample was pumped at a flow rate of 4 μ l/min by a 140A solvent delivery system (Applied Biosystems, Foster City, CA, U.S.A.). The *m/z* values were scanned from 800 to 1600 Da for 9 s; scans were accumulated for 2.5 min. Measurements were calibrated with horse heart myoglobin (16951.5 Da).

RESULTS

Construction of pIPGH1

Plasmid pIN-III-*ompA3* (Ghrayeb et al., 1984) was used as the starting material for constructing a rec-hPGH secretion vector. This plasmid contains a hybrid *lpp/lacPO* promoter and *lacI* gene. A foreign gene can be cloned into the *EcoRI*–*BamHI* multiple cloning site (Figure 1). To insert *hGH-V* cDNA into the pIN-III-*ompA3* vector, we first removed a 90-bp fragment of this vector with *XbaI* and *EcoRI* endonucleases. After purification, the 90-bp fragment was digested with *HhaI*, yielding the 78-bp *XbaI*–*HhaI* sequence containing the 5' region coding for the signal peptide. From *hGH-V* cDNA, a 200-bp fragment was removed with *ThaI* endonuclease. A DNA duplex named A1, with the complementary *HhaI* site at its 5' end and the blunt-end site at the 3' end, was synthesized. The A1 duplex was inserted between a 78-bp fragment of pIN-III-*ompA3* (5' end) and the 606-bp fragment of *hGH-V* cDNA (3' end). The resulting sequence containing the coding region of the *ompA* signal peptide linked to the coding sequence of mature hPGH (Figure 2) was inserted into the *XbaI*–*EcoRI* site of the pIN-III-*ompA3* plasmid. The ligation mixture was used to transform *E. coli* D1210. The correct plasmid, when digested with *XbaI* and *EcoRI*, yielded two fragments (7.4 and 0.74 kb). The plasmids of nine positive colonies were analysed by Southern blotting with 5' ³²P-labelled oligonucleotide A1. The junction between the *ompA* signal coding sequence and the *hGH-V* cDNA was checked by sequence analysis (Sanger et al., 1977). Four positive colonies, each containing the correct plasmid (named pIPGH1), were tested for their capacity to produce, upon induction, soluble rec-hPGH in the periplasm. This was done by immunoassay detection of extracted rec-hPGH (Figure 3). All four colonies were found to produce soluble rec-hPGH. Plasmid pIPGH1 was used for further study. *E. coli* D1210 harbouring pIPGH1 was grown and induced as described above.

Optimization of expression of the *ompA/hGH-V* gene in *E. coli*

We investigated the effects of time and temperature at a low IPTG concentration (50 μ M) on the expression of the *ompA/hGH-V* gene in *E. coli*. This preliminary study was performed in the Multigen convertible bench-top culture apparatus as described in the Experimental section. The concentrations of periplasmic rec-hPGH and total periplasmic protein were measured respectively by radioimmunoassay (Figure 3) and by the Bradford assay (Bradford, 1976). At 37 °C, the relative

Figure 1 Construction of the pIPGH1 secretion-expression vector

Abbreviations: Ba, *Bam*HI; Ec, *Eco*RI; Hh, *Hha*I; Hi, *Hind*III; Xb, *Xba*I; Ps, *Pst*I and Th, *Tha*I.

concentration of rec-hPGH was maximal (2.1 %) after 2 h, decreasing thereafter to 0.8 %. At 30 °C, however, the relative rec-hPGH concentration increased to 2.7 % in 8 h (Figure 4).

Extraction, purification and characterization of rec-hPGH

The induced cells were fractionated, yielding a membrane/cytoplasmic fraction and a periplasmic fraction; 10 % of

the rec-hPGH remained in the membrane/cytoplasmic fraction. The rec-hPGH was purified from the periplasmic fraction as described in the Experimental section. In anion-exchange chromatography, rec-hPGH eluted between 23 % and 42 % $\text{NH}_4\text{HCO}_3/\text{NH}_3$ (Figure 5a). Its purity was as high as 70 %, as determined by SDS/PAGE (Figure 5b). To obtain a homogeneous preparation, the eluted fraction containing rec-hPGH was subjected to gel filtration. As shown in Figure 6, the rec-

1
 CTGATACGAGGGCAAAA ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG
 Met Lys Lys Thr Ala Tle Ala Tle Ala Val Ala Leu
 20
 GCT GGT TTC GCT ACC GTH GCG GCG TTC CCA ACC ATT CCC TTA TCC AGG CTT
 Ala Gly Phe Ala Thr Val Ala Gln Ala Phe Pro Thr Ile Pro Leu Ser Arg Leu
 30
 TTT GAC AAC GCT ATG CTC GCG GCG GCT GCG CTG TAC CAG CTG GCA TAT GAC ACC
 Phe Asp Asn Ala Met Leu Arg Ala Arg Arg Leu Tyr Gln Leu Ala Tyr Asp Thr
 40
 TAT CAG GAG TTT GAA GAA GCG TAT ATC CTG AAG GAG CAG AAG TAT TCA TTC CTG
 Tyr Gln Glu Phe Glu Gln Ala Tyr Ile Leu Lys Glu Gln Lys Tyr Ser Phe Leu
 50
 CAG AAC CCC CAG ACC TCC CTC TGC TTC TCA GAG TCT ATT CCA ACA CCT TCC AAC
 Gln Asn Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn
 60
 AAG GTG AAA ACG CAG CAG AAA TCT AAC CTA GAG CTG CTC GCG ATC TCC CTG CTG
 Arg Val Lys Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu
 70
 CTC ATC CAG TCA TGG CTG GAG CCC GTG CAG CTC CTC AAG AGC GTC TTC GCC AAC
 Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Leu Leu Arg Ser Val Phe Ala Asn
 80
 AAG CTG GTG TAT GGC GCG TCG GAC AGC AAC GTC TAT CAG CAC CTG AAG GAC CTA
 Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn Val Tyr Arg His Leu Lys Asp Leu
 90
 GAG GAA GGC ATC CAA ACG CTG ATG TGG AAG CTG GAA GAT GGC AGC CCC CGG AC
 Glu Glu Gly Ile Gln Thr Leu Met Trp Arg Leu Glu Asp Gly Ser Pro Arg Thr
 100
 GGG CAG ATC TTC AAT CAG TCC TAC AGC AAG TTT GAC ACA AAA TCG CAC AAC GAT
 Gly Gln Ile Phe Asn Gln Ser Tyr Ser Lys Phe Asp Thr Lys Ser His Asn Asp
 110
 GAC GCA CTG CTC AAG AAC TAC GGG CTG CTC TAC TGC TTC AAG AAG GAC ATG GAC
 Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met Asp
 120
 AAG GTC GAG ACA TTC CTG GCG ATC GTG CAG TGC GCG TCT GTG GAG GGC AGC TGT
 Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly Ser Cys
 130
 GGC TTC TAG CCCGGGTGGCATCCCTGTGACCCCTCCCGATGCTCTCTCTGCTGCTGGAGG
 Gly Phe STOP
 140
 TGCTACTCCAGTGGCCCCGCCCCGAAATTC

Figure 2 The primary structure of the ompA/hGH-V protein

(a) The nucleotide sequence of the *ompA/hGH-V* gene and the deduced amino acid sequence of its product. The *ompA* signal peptide is underlined and the cleavage site indicated by an arrow. (b) Hydropathy plot of the *ompA/hGH-V* protein. The vertical line delimits the N-terminal 21-AA transmembrane sequence corresponding to the *ompA* signal peptide.

hPGH preparation appeared homogeneous and the apparent molecular mass of the protein was 22 kDa. We also purified a second form of rec-hPGH, with an apparent molecular mass of 24 kDa (Figure 6). This form dissociates under reducing conditions into two forms (5.7 and 16 kDa; data not shown).

Figure 2 shows the complete nucleotide sequence of the *ompA/hGH-V* hybrid gene. By comparing the 5' portion of the sequence with the signal peptide cleavage site (von Heijne, 1986), amino acid residue 22 (Phe) from the initiation site was tentatively assigned as the first N-terminal amino acid of the mature protein. As determined by amino acid sequencing, the apparently 24 kDa and 22 kDa forms have the same primary structure. The former, however, is nicked between amino acids 141 and 142, owing to

Figure 3 Immunoreactivity of rec-hPGH with 5B4 (a) and K24 (b) mAbs, 125 I-hGH being used as a tracer

hGH (■) and rec-hPGH (□) are added as unlabelled hormones.

Figure 4 Effect of temperature on *ompA/hGH-V* gene expression in *E. coli* D1210

The rec-hPGH concentration is expressed as a percentage of the total periplasmic protein.

the simultaneous occurrence of the sequence Ser, Tyr, Ser, Lys (starting from position 142), after Gln-141. The nicked form migrates more slowly than the intact form in SDS/PAGE. The theoretical pI of hPGH is 8.24, matching well the experimental value (8.2–8.3). The precise molecular mass of rec-hPGH, 22320.4 Da, was determined by the recently introduced technique called electrospray m.s. (Fenn et al., 1989). This result differs by only 1 Da from the molecular mass (22321.4) calculated for the

Figure 5 (a) Sepharose fast-flow ion-exchange chromatography of the $(\text{NH}_4)_2\text{SO}_4$ precipitate

The conditions of chromatography are described in the text. The bars indicate fractions combined to form pools I and II. (b) SDS PAGE of fractions corresponding to pools I (lanes 5–10) and II (lanes 11–14). Pituitary GH (lane 1); molecular-mass marker (lane 2); lysate before (lane 3) and after induction (lane 4).

Figure 6 SDS/PAGE of the exclusion-chromatography fractions

Lanes a, b and c show respectively GH, rec-hPGH (22 kDa form), and a fraction enriched in rec-hPGH (form exhibiting a 24 kDa apparent molecular mass). Lanes d, e and f show the same samples after 2-mercaptoethanol treatment. Lanes g, h, i, j, k and l show the immunoblots corresponding respectively to a, b, c, d, e and f. The anti-GH 5B4 mAb was used as the specific antibody.

reduced form. Since rec-hPGH has been shown to exist in an oxidized form containing two disulphide bridges, we checked the molecular mass after addition of dithiothreitol. Figure 7(b)

Figure 7 Electrospray mass spectrum of the rec-hPGH

The values marked '+' refer to the number of charges for particular values of m/z . The inserts are the molecular-mass spectra of the protein as calculated by the 'conversion programme' supplied with the instrument. The top figure (a) represents the native protein, the bottom figure (b) is the mass spectrum obtained after reduction with dithiothreitol for 30 min. The low-mass peak at 22175.5 corresponds to a molecular species of the protein lacking the N-terminal Phe residue. The mass value for the higher-molecular-mass minor peak at 22413.6 remains unexplained.

clearly shows that this treatment increases the molecular mass by 4.2 Da.

The immunoreactivity of rec-hPGH with 5B4 and K24 mAbs was compared with that of pituitary GH (Figure 3) and found to be similar to that of the natural product extracted from full-term placenta (Frankenne et al., 1988).

DISCUSSION

In preliminary experiments (Igout et al., 1989) we have demonstrated that the pIPGH1/*E. coli* D1210 system expresses rec-hPGH and transports it into the periplasm. However, rec-hPGH accounted for only 0.1% of the total periplasmic protein. To avoid excessive expression of the chimeric gene and formation of inclusion bodies or rapid degradation of rec-hPGH, induction was performed at 30 °C instead of 37 °C and at low IPTG concentrations (e.g. 50 μM). Loss of rec-hPGH by precipitation near its pI (8.24) was avoided by forming spheroplasts at pH 7.2 instead of pH 8 as described by Koshland and Botstein (1980).

Under these conditions, the relative rec-hPGH concentration increased 20-fold and the protein remained soluble, but the hGH-V cDNA expression remains significantly lower than that of hGH-N with the same expression system (Hsiung et al., 1986). It had to be concentrated in 8 M urea, however, to avoid aggregation and precipitation.

The *E. coli* D1210/pIPGH1 system also secreted, under non-optimal conditions, a small amount of a second form with an

apparent molecular mass of 24 kDa. This form was identical to the 22 kDa form but was cleaved between amino acids 141 and 142. The disulphide bond between Cys-53 and Cys-165 maintains the association of the two resulting peptides. This type of cleavage has previously been observed for human growth hormone extracted from the pituitary gland (Lewis et al., 1976). Treatment with protease inhibitor (EDTA, PMSF and Trasylol) does not protect rec-hPGH from partial cleavage between amino acids 141 and 142. The ompA signal peptide was correctly processed: this sequence only has the ability to anchor the nascent polypeptide to the cytoplasmic membrane (Figure 2).

The pI of rec-hPGH, both as calculated from the sequence and as experimentally determined, is 8.24 and not 9.3 as suggested by Cooke et al. (1988). Preliminary experiments show that rec-hPGH binds to the hepatic GH receptor (Igout et al., 1991), confirming its correct folding and potential somatogenic activity. Sufficient quantities of rec-hPGH are now available for exhaustive biological testing and pharmacological studies.

We wish to thank Dr. M. J. Inoye for the gift of pIN-III-ompA3, Dr. Gellerfors from KabiVitrum (Sweden) for the gift of recombinant hGH-N, and M. McNamara for critically reading the manuscript. J.V. thanks the Fund for Joint Basic Research (contract 32.001.91) for financial support. This work was supported by grants from the 'Région Wallonne' (grant no. 1532) and from the EEC (SCI-CT91-0759-TSTS).

REFERENCES

- Barsh, G. S., Seeburg, P. H. and Gelinas, R. E. (1983) *Nucleic Acids Res.* **83**, 3939–3959
- Bjellquist, B. and Ek, K. (1982) LKB-Produkter AB, Bromma, Application note 321
- Bolander, F. F. (1989) in *Molecular Endocrinology*, pp. 25–26, Academic Press, San Diego, CA
- Bolivar, F. and Backman, K. (1979) *Methods Enzymol.* **68**, 245–267
- Boyer, H. W. and Rouland-Dussoix, D. (1969) *J. Mol. Biol.* **41**, 459–472
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248
- Chen, E. Y., Liao, Y. C., Smith, D. H., Barrera-Saldana, H. A., Gelinas, R. E. and Seeburg, P. H. (1989) *Genomics* **4**, 479–497
- Cooke, N. E., Ray, J., Emery, J. G. and Liebha, S. A. (1988) *J. Biol. Chem.* **263**, 9001–9006
- Eriksson, L., Frankenke, F., Eden, S., Hennen, G. and von Schoultz, B. (1989) *Br. J. Obstet. Gynaecol.* **96**, 949–953
- Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. and Whitehouse, C. M. (1989) *Science* **246**, 64–71
- Frankenne, F., Closset, I., Gomez, F., Scippo, M. L., Small, J. and Hennen, G. (1988) *J. Clin. Endocrinol. Metab.* **66**, 1171–1180
- Frankenne, F., Scippo, M. L., Van Beeumen, J., Igout, A. and Hennen, G. (1990) *J. Clin. Endocrinol. Metab.* **71**, 15–18
- Ghrayeb, J., Kimura, H., Takahara, M., Hsiung, H., Msaii, Y. and Inoye, M. J. (1984) *EMBO J.* **3**, 2437–2442
- Gomez, F., Pirens, G., Schaus, C., Closset, J. and Hennen, G. (1984) *J. Immunoassay* **5**, 145–157
- Hennen, G., Frankenke, F., Closset, J., Gomez, F., Pirens, G., Smal, J., Beckers, A., El Khayat, N. and Lambotte, R. (1985a) in *Serono Symp. Publ.* Raven Press, **30**, 29–40
- Hennen, G., Frankenke, F., Pirens, G., Gomez, F., Closset, J. and El Khayat, N. (1985b) *Lancet* **16**, 399
- Hennen, G., Frankenke, F., Closset, J., Gomez, F., Smal, J. and El Khayat, N. (1986) *Bull. Acad. R. Belgique* **141**, 111–119
- Hirt, H., Kimelman, J., Birnbaum, M. J., Chen, E. Y., Seeburg, P. H., Eberhardt, N. L. and Barta, A. (1987) *DNA* **6**, 59–70
- Hsiung, H. M., Mayne, G. N. and Becker, G. W. (1986) *Bio/Technology* **4**, 991–995
- Igout, A., Scippo, M. L., Frankenke, F. and Hennen, G. (1988) *Arch. Int. Physiol. Biochem.* **96**, 63–67
- Igout, A., Scippo, M. L., Frankenke, F. and Hennen, G. (1989) *Nucleic Acids Res.* **17**, 3998
- Igout, A., Frankenke, F., Lhermitte-Balleriaux, M. F., Scippo, M. L. and Hennen, G. (1991) 73rd Annu. Meet. Endocrine Soc., Abstr. 1308, pp. 357
- Jara, C. S., Salud, A. T., Bryant-Greenwood, G. D., Pirens, G., Hennen, G. and Frankenke, F. (1989) *J. Clin. Endocrinol. Metab.* **69**, 1069–1072
- Koshland, D. and Botstein, D. (1980) *Cell* **20**, 749–760
- Kyte, J. and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lewis, U. J., Singh, R. N. P., Peterson, S. M. and Vanderlaan, W. P. (1976) in *Growth Hormone and Related Peptides* (Pecile, A. and Muller, E. E., eds.), pp. 64–74, Excerpta Medica, Amsterdam
- Osborn, M. J., Gonder, J. E., Parisi, E. and Carson, J. (1972) *J. Biol. Chem.* **247**, 3962–3972
- Owerbach, D., Rutter, W. J., Martial, J. A., Baxter, J. D. and Shows, T. B. (1980) *Science* **209**, 289–292
- Salder, J. R., Tecklenburg, M. and Bety, J. L. (1980) *Gene* **8**, 279–300
- Sanger, F., Niclen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Scippo, M. L., Frankenke, F., Hooghe-Peeters, E., Igout, A., Velkeniers, B. and Hennen, G. (1993) *Mol. Cell. Endocrinol.* **92**, R7–R13
- Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Von Heijne, G. (1986) *Nucleic Acids Res.* **14**, 4683–4690