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Structural requirements for α-mating factor activity

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Abstract The sexual hormone of *S. cerevisiae*, α-mating factor (α-MF, WHWLQLKPGQPMY) has structural homology with mammalian luteinizing hormone releasing hormone (LHRH, pEHWSYGLRPG-NH_2) and has been shown to exhibit LHRH activity [Loumaye et al. (1982) Science 218, 1323–1325]. We have tested whether LHRH has α-MF activity in yeast and found that it does not. We therefore synthesized a series of hybrid peptides of α-MF and LHRH to study the structural features which determine α-MF and LHRH activities. A hybrid peptide consisting of the LHRH sequence with the C-terminal tetrapeptide (QPMY) of α-MF did not exhibit α-MF activity. Thus, the lack of α-MF activity of LHRH is not due solely to the absence of the C-terminal residues. Substitution of Lys^7 in α-MF with Arg, as is found in LHRH, did not affect the α-MF activity, nor did an additional substitution of Trp^1 with pGlu. However, the C-terminal four amino acids of α-MF were necessary for α-MF activity. Our results indicate that insertion of a Ser residue in position 4 as found in LHRH abolishes α-MF activity. These results suggest that, in addition to an intact C-terminus, correct spacing of the N-terminal His^2 and the C-terminus is required for α-MF activity. The hybrid peptides all exhibited less LHRH activity than either LHRH or α-MF. These structure-function studies indicate that the structural homology between these two reproductive hormones may not reflect an evolutionary relationship between them.

Key words: Peptide; Mating factor; Yeast; LHRH; Structure/function; Activity

1. Introduction

Comparative studies of signal transduction in mammalian cells and yeasts have revealed a significant structural and functional conservation of the components involved. The unicellular yeasts *S. pombe* and *S. cerevisiae* are therefore useful models for studying several cellular mechanisms including growth control and differentiation [1]. The budding yeast *S. cerevisiae* exists in one of two mating types, **a** and α, which can mate with each other [2]. During the mating process the cells communicate by excreting diffusible pheromones, α-mating factor (α-MF) or **a**-mating factor. The cells respond to the mating factors by elongating towards the pheromone source, by altered patterns of gene transcription and by G_1 arrest of cell division. The mating factors are thus negative growth factors in yeast. Since many cellular signaling systems in mammals and yeasts are homologous the latter are useful for elucidating specific parts of these systems. As shown in Table 1, *S. cerevisiae* α-MF is homologous to mammalian LHRH and has been found to exhibit some LHRH activity [3]. Since the receptors for these hormones both belong to the seven transmembrane family of G protein-coupled receptors [4–6], structure/function studies of the hormones might yield information about receptor binding and activation, which could be important in the design of LHRH agonists and antagonists. Apart from its function in LH release, LHRH has been found to have growth inhibitory effects on several cell lines [7–10] and thus shares with α-MF the property of growth inhibition/differentiation [11–14]. We have explored the structural similarity of LHRH and α-MF in more detail by testing LHRH for α-MF activity and by synthesizing hybrids of LHRH and α-MF, and testing the hybrids for α-MF activity and for binding to a mammalian LHRH receptor.

2. Materials and methods

All chemicals were of analytical grade or higher quality.

Peptides, synthesized by the solid-phase method, LHRH, and buserelin ([D-Ser^6(t-Bu),des-Gly^{10},Pro^9NHEt]-LHRH) were from Peptech Europe (Copenhagen, Denmark). α-mating factor was from Sigma (St. Louis, USA).

S. cerevisiae strain MT502 (genotype: *Mata sst2- leu2-3, 112 met1 his3 and/or 6 can1*) was kindly provided by Egel-Mitani et al. [15].

Amino acid analysis was performed as described by Barkholt and Jensen [16].

Mass spectrometry was carried out on a Bio-Ion model 20 mass spectrometer operated at 13 kV accelerating voltage. Samples were applied to nitrocellulose-coated targets and air-dried before analysis.

Assay for α-mating factor activity: The strain MT502 was grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) to a density of 10^7 cells/ml. 0.1 ml of cells was mixed with 4 ml of 0.7% YPD agar (at 42°C) and spread on a YPD plate. After the agar had solidified, discs of filter paper (5 mm diameter) were placed on the top agar and 10 μl of peptide (1 mg/ml) was added to each disc. After incubation overnight the plates were inspected, and the diameter of the growth inhibition zone was measured.

Assay for binding to the LHRH receptor: Binding assays were performed as described by Millar et al. [17] using αT3 gonadotrope cell membranes. Briefly, cells were detached from the culture dishes in HEPES binding buffer (10 mM HEPES, 1 mM EDTA, pH 7.4), homogenized with a Dounce homogenizer and centrifuged at 10000×*g* for 40 min at 4°C. The crude membrane pellet was resuspended in binding buffer and incubated with approx. 50000 cpm ^{125}I-[D-Ala^6,*N*-Me-Leu^7,Pro^9-NHEt]-LHRH and 10^{-5} M test peptide for 60 min on ice to achieve equilibrium. The incubation was terminated by addition of 3 ml 0.01% aqueous polyethyleneimine and immediate filtration through glass-fiber filters (GF/C, Whatman) presoaked in 1% polyethyleneimine. The filters were washed twice with 0.01% polyethyleneimine and bound radioactivity was determined by scintillography.

3. Results

Tables 1 and 2 list the structures and amino acid analyses of the peptides synthesized and tested for activity, while Fig. 1 shows representative mass spectrograms of commercial α-MF

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and one of the hybrid peptides. All the hybrid peptides were purified by preparative HPLC after synthesis, and amino acid analyses were in agreement with the theoretically expected values. In Fig. 2 are shown the results of α-MF activity experiments. LHRH did not show α-MF activity and neither did buserelin, an analogue of LHRH (Fig. 2A). A hybrid peptide, αLHRH, consisting of the four C-terminal amino acids of α-MF added to LHRH did not exhibit α-MF activity (Fig. 2B), and αLHRH, furthermore, did not inhibit the biological activity of α-MF in competition assays (Fig. 2C). Compared to α-MF, the αLHRH hybrid molecule has the same C-terminal sequence (Pro-Gly-Gln-Pro-Met-Tyr) and identical residues at positions 2 and 3 (His-Trp). However, it has an N-terminal pGlu instead of Trp, an extra amino acid separating the His-Trp sequence from the C-terminal part of the molecule, a Tyr-Gly sequence instead of a Leu-Gln sequence in the middle of the molecule and arginine instead of lysine. Any one of these differences could account for the lack of α-MF activity of αLHRH and this was addressed by synthesis of further hybrid peptides (Table 1). An analogue of α-MF, αLHRH-1, in which Lys^7 was substituted with Arg exhibited full mating factor activity (Fig. 2D, Table 3). Thus, the conservative substitution does not impair the biological activity. The peptide αLHRH-2 which contained an additional change of the N-terminal Trp to pGlu also retained α-MF activity (Table 3). Consequently, the N-terminal charge and the Trp^1 side chain are unimportant for mating factor activity. A further change of Gln to Gly at position 5 relative to α-MF in the peptide αLHRH-3 also did not affect the mating factor activity (Fig. 2D, Table 3). This peptide differs from the inactive αLHRH peptide only in the absence of Ser^4 and in the conservative substitution of Leu for Tyr. Therefore, the insertion of Ser^4 of LHRH probably accounts for the inactivity of αLHRH. Deletion of the C-terminal tetrapeptide (Gln-Pro-Met-Tyr) of αLHRH-3 in αLHRH-4 completely abolished mating factor activity (Fig. 2D, Table 3).

Fig. 3 shows the results of receptor binding experiments. 10^{-5} M α-MF inhibited binding of the labelled LHRH agonist by 60.9% in membranes prepared from the αT3 mouse gonadotroph cell line. This is in close agreement with the published IC_{50} of 4.5×10^{-5} M [3]. The other peptides showed less inhibition of LHRH agonist binding with no obvious correlation between structure and activity. αLHRH and αLHRH-3 both showed around 50% inhibition of LHRH agonist binding, while αLHRH-2 and αLHRH-4 both showed approx. 30% inhibition of agonist binding. αLHRH-1 was the least active peptide with no significant inhibition at the concentration used.

Table 1
Names and structures of the peptides synthesized and tested

Peptide	Structure
LHRH	pGluHisTrpSerTyrGlyLeuArgProGlyNH_2
α-MF	TrpHisTrp---LeuGlnLeuLysProGlyGlnProMetTyr
αLHRH	pGluHisTrpSerTyrGlyLeuArgProGlyGlnProMetTyr
αLHRH-1	TrpHisTrp---LeuGlnLeuArgProGlyGlnProMetTyr
αLHRH-2	pGluHisTrp---LeuGlnLeuArgProGlyGlnProMetTyr
αLHRH-3	pGluHisTrp---LeuGlyLeuArgProGlyGlnProMetTyr
αLHRH-4	pGluHisTrp---LeuGlyLeuArgProGlyNH_2

To facilitate comparison a gap (- - -) has been introduced in some of the peptides. The three-letter abbreviations for the amino acids are used. pGlu denotes pyroglutamic acid and $GlyNH_2$ denotes the C-terminal glycine amide.

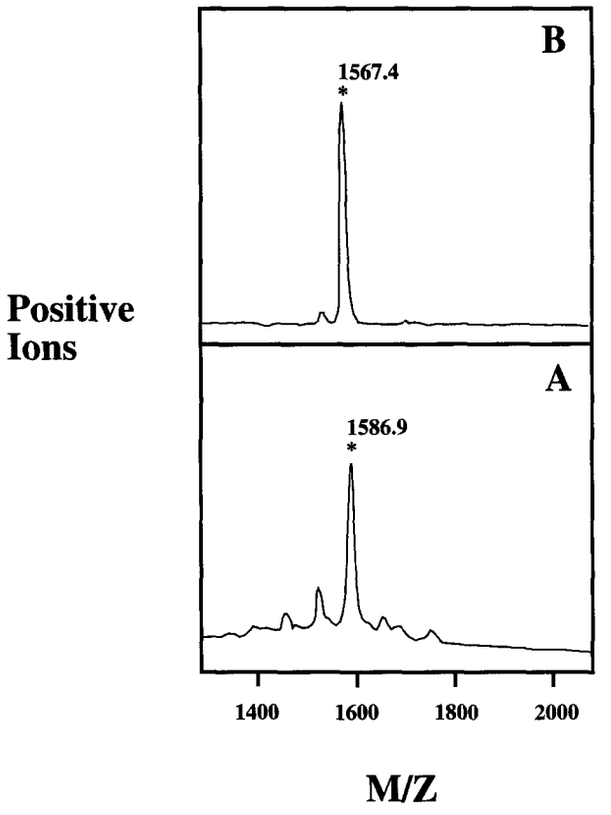

Fig. 1. Representative mass spectrograms of commercial α-MF (A) and one of the hybrid peptides, αLHRH-3 (B), illustrating the purity of the peptides used.

Table 3 summarizes the results from the activity experiments.

4. Discussion

LHRH was found to be inactive in the α-MF activity assay and a peptide consisting of the LHRH sequence plus a C-terminal extensioncorresponding to the C-terminus of α-MF was also inactive. This shows that the inactivity of LHRH was not solely due to the absence of the C-terminal residues of α-MF. Therefore, we made hybrid peptides consisting of α-MF with progressively more amino acids characteristic of LHRH in order to assess which residues are necessary for α-MF activity and which residues of LHRH decrease α-MF activity.

Table 2
Amino acid analysis of peptides used in this study and calculated and experimentally determined masses of the peptides

	αLHRH	αLHRH-1	αLHRH-2	αLHRH-3	αLHRH-4
Glu	1.5 (1)	2.3 (2)	3.4 (3)	2.2 (2)	1.2 (1)
Pro	2.2 (2)	2.1 (2)	2.3 (2)	2.2 (2)	1.1 (1)
Gly	1.9 (2)	1.5 (1)	1.1 (1)	2.2 (2)	2.2 (2)
Met	–	1.4 (1)	1.4 (1)	1.5 (1)	–
Leu	1.0 (1)	2.0 (2)	2.0 (2)	2.0 (2)	2.0 (2)
Tyr	2.1 (2)	1.0 (1)	1.0 (1)	1.0 (1)	–
His	1.1 (1)	0.8 (1)	0.8 (1)	1.0 (1)	1.1 (1)
Trp	0.8 (1)	1.6 (2)	1.6 (2)	1.0 (1)	0.7 (1)
Arg	1.0 (1)	1.0 (1)	1.0 (1)	1.1 (1)	1.2 (1)
Exp. mass	1703.3	1712.5	1638.3	1567.4	1047.0
Calc. mass	1702.9	1712.0	1636.9	1565.9	1045.2

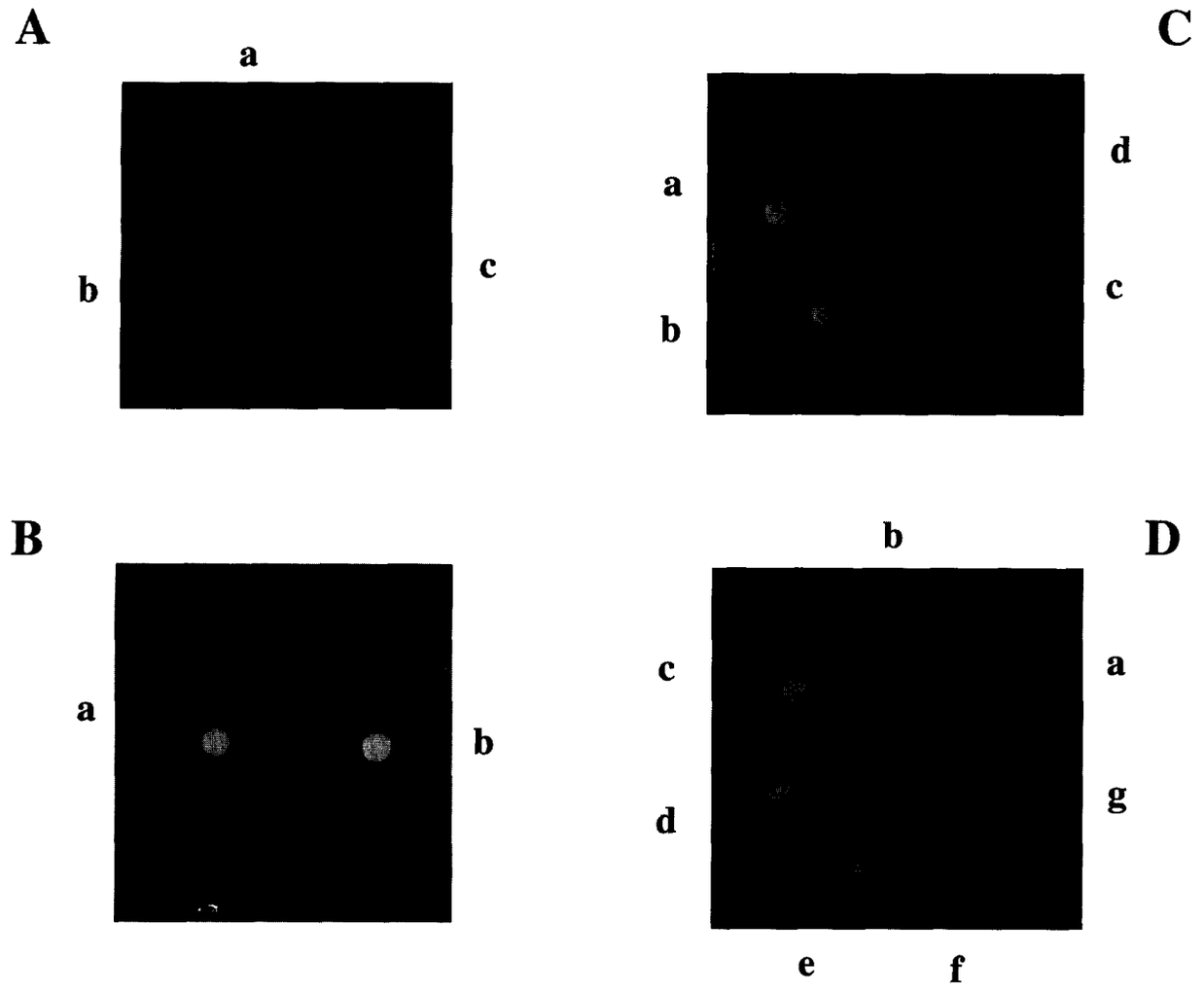

Fig. 2. Results of assay for α-MF activity on yeast strain MT502. (A) Assay of α-MF (a), LHRH (b) and buserelin (c). (B) Assay of α-MF (a) and αLHRH (b). (C) Competition assay between α-MF and αLHRH (1 mg/ml) in the following amounts: 10 μl (a), 5 μl (b), 1 μl (c), none (d). (D) a, α-MF; b, LHRH; c, αLHRH; d, αLHRH-1; e, αLHRH-2; f, αLHRH-3; g, αLHRH-4.

Previously, it was shown that Arg^8 is important for LHRH activity and that substitution with Lys decreased activity approx. 10-fold [18]. We therefore investigated whether the reverse applies to α-MF. However, αLHRH1 had α-MF activity, showing that either basic residue is tolerated in α-MF. Additional substitution of Trp^1 with pGlu also did not affect α-MF activity, in agreement with previous reports that Trp^1 is not important for α-MF activity [19]. This is in contrast to LHRH where the N-terminal pGlu is important for activity [20]. Substitution of Gln^5 with the achiral Gly which is important for LHRH activity [21] did not affect α-MF activity,

Table 3
Biological activities of the peptides tested in this study

Peptide	α-MF activity	LHRHR binding (% inhibition)
LHRH	–	100
α-MF	+	60.9
αLHRH	–	46.4
αLHRH-1	+	6.1
αLHRH-2	+	29.9
αLHRH-3	+	47.8
αLHRH-4	–	31.9

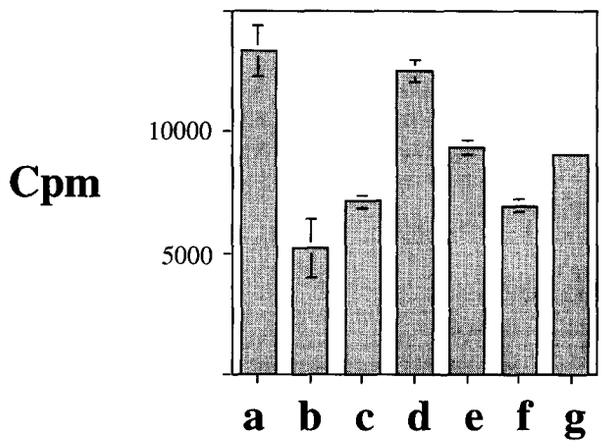

Fig. 3. Histogram showing the results of testing the peptides for their ability to compete with an LHRH agonist for binding to the mammalian LHRH receptor. The figure shows the mean of triplicate determinations of LHRH agonist binding to the LHRH receptor in the absence (a) or presence of competing peptides (b–g). a, control; b, α-MF; c, αLHRH; d, αLHRH-1; e, αLHRH-2; f, αLHRH-3; g, αLHRH-4.

Table 4
Structures of vertebrate LHRH molecules and yeast α-mating factors (α-MF)

Peptide	Sequence	References
Mammalian LHRH	pEHWSYGLRPGNH$_2$	[22,23]
Chicken I LHRH	pEHWSYGLQPGNH$_2$	[24–27]
Chicken II LHRH	pEHWSHGWYPGNH$_2$	[28]
Salmon LHRH	pEHWSYGWLPGNH$_2$	[29]
Catfish LHRH	pEHWSHGLNPGNH$_2$	[30]
Dogfish LHRH	pEHWSHGWLPGNH$_2$	[31]
Lamprey I LHRH	pEHYSLGWKPGNH$_2$	[32]
Lamprey III LHRH	pEHWSHDWKPGNH$_2$	[33]
Common for LHRH	pEH S PG	
S. cerevisiae α-MF	WHW-LQLKPGQPMY	[19]
S. klyuverii α-MF	WHW-LSFSKGEPMY	[34,35]
Common for α-MF	WHW-L G PMY	
Common for active hybrid peptides and α-MF	HW-L G PMY	
Common for all	H G	

The one-letter abbreviations for the amino acids are used.

indicating that neither the L-stereochemistry, nor the amide side chain is important for α-MF activity. Deletion of the C-terminal three residues of this peptide or insertion of a Ser in position 4 abolished α-MF activity. Together these two results confirm previous findings that the C-terminal residues of α-MF are important for its activity [19] and show that the relative positions of the two ends of the molecule are important for activity.

Analysis of the LHRH receptor binding activity of the hybrid peptides did not support a functional relationship between the two peptides. We replicated the previously reported LHRH receptor binding activity of α-MF. However, none of the hybrid peptides exhibited LHRH receptor binding activity intermediate between those of LHRH and α-MF, and indeed all of the hybrid peptides were less active than α-MF.

A comparison of the structures of sexual hormones from different species (Table 4) points to a conserved role of the pGlu^1,His^2, Ser^4, and Gly^{10} for LHRH activity. In addition, position 3 is Trp in all LHRH variants except one where it is Tyr, and position 6 is Gly in all except one. Comparison of α-mating factors and the active hybrid peptides shows conservation of His^2, Trp^3, Leu^4, Gly^9, Pro^{11}, Met^{12}, and Tyr^{13}. Thus, the only conserved amino acid in all the peptides listed in Table 2 is His^2 and this residue has been shown to be important for activity of both α-MF and LHRH [19,36,37]. However, the results presented here on the structure/activity relationships of α-MF are significantly different from the reported structure/activity relationships of LHRH. Thus, there is very little conservation of function between the two peptides and the homology between the peptides may not reflect an evolutionary relationship.

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References

[1] Botstein, D. and Fink, G.R. (1988) Science 240, 1439–1442.
[2] Herskowitz, I., Rine, J. and Strathern, J.N. (1992) In The Molecular and Cellular Biology of the Yeast *Saccharomyces*. Vol. 2. Gene Expression (Jones, E.W., Pringle, J.R. and Broach, J.R. eds.) pp. 583–656, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[3] Loumaye, R., Thorner, J.B. and Catt, K.J. (1982) Science 218, 1323–1325.
[4] Dohlman, H.G., Thorner, J., Caron, M.G. and Lefkowitz, R.J. (1991) Annu. Rev. Biochem. 60, 653–688.
[5] Jackson, C.L., Konopka, J.B. and Hatwell, L.H. (1991) Cell 67, 389–402.
[6] Hargrave, P.A. (1991) Curr. Opin. Struct. Biol. 1, 575–581.
[7] Liebow, C., Lee, M.T., Kamer, A.R. and Schally, A.V. (1991) Proc. Natl. Acad. Sci. USA 88, 2244–2248.
[8] Fekete, M., Zalatnai, A. and Schally, A.V. (1989) Cancer Lett. 45, 87–91.
[9] Scambia, G., Panici, P.B., Baiocchi, G., Perrone, L., Gagini, C., Iacobelli, S. and Mancuso, S. (1988) Anticancer Res. 8, 187–190.
[10] Miller, W.R., Scott, W.N., Morris, R., Fraser, H.M. and Sharpe, R.M. 1985) Nature 313, 231–233.
[11] Cross, F., Hartwell, L.H., Jackson, C. and Konopka, J.B. (1988) Annu. Rev. Cell. Biol. 4, 429–457.
[12] Herskowitz, I. (1989) Nature 342, 749–757.
[13] Chang, F. and Herskowitz, I. (1990) Cell 63, 999–1011.
[14] Chan, R.K. and Otte, C.A. (1982) Mol. Cell. Biol. 2, 21–29.
[15] Egel-Mitani, M., Flygenring, H.P. and Hansen, M.T. (1990) Yeast 6, 127–137.
[16] Barkholt, V. and Jensen, A.L. (1989) Anal. Biochem. 177, 318–322.
[17] Millar, R.P., Davidson, J., Flanagan, C. and Wakefield, I. (1995) Methods Neurosci. 25, 145–162.
[18] Geiger, R., König, W., Sandow, J. and Schally, A.V. (1974) Hoppe Seyler's Z. Physiol. Chem. 355, 1526–1534.
[19] Masui, Y., Chino, N., Sakakibara, S., Tanaka, T., Murakami, T. and Kita, H. (1977) Biochem. Biophys. Res. Commun. 78, 534–538.
[20] Rivier, J., Amoss, M., Rivier, C. and Vale, W. (1974) J. Med. Chem. 17, 230–233.
[21] Coy, D., Vilchez-Martinez, J.A., Coy, E., Nishi, M., Arimura, A. and Schally, A.V. (1975) Biochemistry 14, 1848–1851.
[22] Matsuo, H., Baba, Y., Nair, R.M., Arimura, A. and Schally, A.V. (1971) Biochem. Biophys. Res. Commun. 43, 1334–1439.
[23] Amoss, M., Burgus, R., Blackwell, R., Vale, W., Fellows, R. and Guillemin, R. (1971) Biochem. Biophys. Res. Commun. 44, 205–210.
[24] King, J.A. and Millar, R.P. (1982) J. Biol. Chem. 257, 10722–10728.
[25] King, J.A. and Millar, R.P. (1982) J. Biol. Chem. 257, 10729–10732.
[26] Miyamoto, K., Hasegawa, Y., Minegishi, T., Nomura, M., Takahashi, Y., Igarashi, M., Kangawa, K. and Matsuo, H. (1982) Biochem. Biophys. Res. Commun. 107, 820–827.
[27] Miyamoto, K., Hasegawa, Y., Igarashi, M., Chino, N., Sakakibara, S., Kangawa, K. and Matsuo, H. (1983) Life Sci. 32, 1341–1347.
[28] Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K. and Matsuo, H. (1984) Proc. Natl. Acad. Sci. USA 81, 3874–3878.
[29] Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., Rivier, J. and Vale, W. (1983) Proc. Natl. Acad. Sci. USA 80, 2794–2798.

[30] Ngamvongchon, S., Lovejoy, D.A., Fischer, W.H., Craig, A.G., Nahorniak, C.S., Peter, R.E., Rivier, J.E. and Sherwood, N.M. (1992) Mol. Cell. Neurosci. 3, 17–22.
[31] Lovejoy, D.A., Fischer, W.H., Ngamvongchon, S., Craig, A.G., Nahorniak, C.S., Peter, R.E., Rivier, J. and Sherwood, N.M. (1992) Proc. Natl. Acad. Sci. USA 89, 6373–6377.
[32] Sherwood, N.M., Sower, S.A., Marshak, D.R., Fraser, B.A. and Brownstein, M.J. (1986) J. Biol. Chem. 261, 4812–4819.
[33] Stotzler, D., Kiltz, H. and Duntze, W. (1976) Eur. J. Biochem. 69, 397–400.
[34] Egel-Mitani, M. and Hansen, M.T. (1987) Nucl. Acids Res. 15, 6303.
[35] Sakurai, A., Sakamoto, M., Tanaka, H., Esumi, Y., Takahashi, N., Fujimura, H., Yanagishima, N. and Banno, I. (1984) FEBS Lett. 166, 339–342.
[36] Levin, Y., Khare, R.K., Abel, G., Hill, D., Eriotou-Bartioga, E., Becker, J.M. and Naider, F. (1993) Biochemistry 32, 8199–8206.
[37] Schally, A.V. and Coy, D.H. (1977) In Hypothalamic Peptide Hormones and Pituitary Regulation (Porter, C. ed.) pp. 99–121. Plenum, NY, USA.