Influence of Subunit Interactions on Lutropin Specificity

IMPLICATIONS FOR STUDIES OF GLYCOPROTEIN HORMONE FUNCTION*

Laurey Cosowsky, Win Lin, Yi Han, Michael P. Bernard, Robert K. Campbell‡, and William R. Moyle§

From the Department of Obstetrics and Gynecology, Robert Wood Johnson (Rutgers) Medical School, Piscataway, New Jersey 08854

Bovine lutropin (bLH) and human chorionic gonadotropin (hCG) are heterodimeric glycoprotein hormones required for reproduction. Both bind rat LH receptors (rLHRS), but hCG binds human LH receptors (hLHRS) 1000–10,000 fold better than bLH. We tested the premise that this difference in affinity could be used to identify lutropin receptor contacts. Heterodimers containing hCG/bLH α- or β-subunit chimeras that bound hLHR like hCG (or bLH) were expected to have hCG (or bLH) residues at the receptor contact sites. Analogs containing one subunit derived from hCG bound hLHR much more like hCG than bLH, indicating that each bLH subunit contains all the residues sufficient for high affinity hLHR binding. Indeed, the presence of bovine α-subunit residues increased the activities of some hCG analogs. The low hLHR activity of bLH was due primarily to an interaction between its α-subunit and β-subunit residue Leu95. Leu95 does not appear to contact the hLHR since it did not influence the hLHR activity of heterodimers containing human α-subunit. These observations show that interactions within and between the subunits can significantly influence the activities of lutropins, thereby confounding efforts to identify ligand residues that contact these receptors.

The gonadotropins human lutropin (hLH),1 human chorionic gonadotropin (hCG), and human follitropin (hFSH) are essential for reproduction and have been used for many years to enhance human fertility. Development of clinically useful agonist and antagonist analogs would be facilitated by knowledge of how these ligands interacted with their receptors. Two radically different models of gonadotropin-receptor interaction have been proposed (1, 2) based on the crystal structures of hCG (3, 4) and ribonuclease inhibitor (5, 6), a protein containing a leucine-rich repeat motif thought to be similar to those in the glycoprotein hormone receptors. These models could be readily distinguished if the portions of the hormone that contacted their receptors were known.

Like other glycoprotein hormones, the gonadotropins are heterodimers that contain a conserved α-subunit and a hormone-specific β-subunit (7). Each subunit is divided into three large loops by a cysteine knot (3, 4), and the heterodimer is stabilized by a portion of the β-subunit termed the “seat belt” (3) that is wrapped around α-subunit loop 2. Based on the activities of chemically and enzymatically modified hormones (summarized by Pierce and Parsons (7)), synthetic hormone fragments (8–12), and analogs prepared by site-directed mutagenesis (13–19), residues throughout both hormone subunits have been suggested to participate in essential high affinity hormone receptor contacts. Surprisingly, some hCG residues proposed to contact LH receptors are in regions recognized by monoclonal antibodies that bind to hCG-receptor complexes (1, 20). Others thought to be essential for receptor contacts can be replaced without disrupting receptor binding. For example, replacing hCG seat belt residues 101–109 with their hFSH counterparts led to analogs that bound FSH receptors with high affinity (21, 22), even though they contained hCG β-subunit residues at positions thought to interact with the FSH receptor. These findings raise significant questions about the identities of residues that have been proposed to be responsible for high affinity receptor contacts.

Studies described in this report were initiated with the goal of identifying residues in hCG that are essential for high affinity contacts with the human LH receptor. The heterodimeric nature of the glycoprotein hormones confounds efforts to identify residues needed for essential high affinity contact, and with few exceptions (23), neither free subunit has significant biological activity (7). Thus, any modification of the hormone that distorts the interaction between its subunits has the potential to disrupt hormone activity, a problem that has been recognized for many years (7) but largely ignored. In the experiments reported here, we sought to minimize this problem by limiting our studies to lutropins such as hCG and bLH that bind the rat lutropin receptor well, an indication that their overall conformations are very similar. We anticipated that the dramatic differences in the affinities of these hormones for the human lutropin receptor (24, 25) would be caused by a few key residues in the primary receptor contact site that could be identified by comparing the abilities of hCG/bLH chimeras to bind the human and rat receptors. Chimeras that had hCG residues in the contact site were expected to bind the hLHR like hCG; those that had bLH residues in this site were expected to bind to hLHR like bLH. As we show here, the low affinity of bLH for hLHR appears to involve an interaction between its subunits that either distorts the region of the high affinity contact and/or creates a steric interaction between the hormone and the receptor. This implies that subtle changes in hormone conformation can exert a much more dramatic influence on ligand binding than commonly perceived.
Binding of bLH to hLHR

MATERIALS AND METHODS

Purified hCG was obtained from Drs. Robert Canfield and Steven Birken (Columbia University, NY). Purified bLH was obtained from Dr. John Pierce (University of California at Los Angeles, CA). Antibodies were obtained from Dr. Canfield, Drs. Glenn Armstrong and Robert Wolfert (Hybritech Inc., CA), and Dr. Janet Roser (University of California at Davis, CA) as noted previously (1, 20). The hLHR cDNA was obtained from Dr. Aaron Hsu (Stanford University, CA) in a vector that was used without modification to make stable CHO cell lines that express the receptor. These lines were prepared by co-transfecting CHO cells with the hLHR vector and pSV2neo, a vector encoding aminoglycoside phosphotransferase downstream of the 5′SV40 early promoter (26), selecting stable transformants in the presence of 50 μg G418/ml, and identifying receptor-expressing cell lines based on their ability to bind [125I]hCG. The rLHR cDNA (27) was inserted downstream of the metallothionein promoter in a vector (pLEN) kindly supplied by Dr. Peter Kushner (University of California at San Francisco, CA). This was accomplished by cloning the XhoI-BamHI fragment of pSVL-hCG(21) into the unique XhoI-BamHI sites of pLEN to create a vector (pLEN-hCG(21)) that has a unique XhoI site immediately downstream of the XhoI site. The XhoI-BamHI fragment containing the coding region of hCG′ was excised and replaced with an XhoI-BamHI fragment containing the entire rLH receptor cDNA prepared as described earlier (27).

Tables and figures

| α-Subunit | Region of α-Subunit | Potency Relative to hCG |
|-----------|---------------------|------------------------|
| hCG       |                     | 1.00 ± 0.00            |
| bLH       |                     | 0.27 ± 0.00            |
| hL1-6     |                     | 0.57 ± 0.00            |
| hL1-26    |                     | 1.00 ± 0.00            |
| hL1-7     |                     | 0.57 ± 0.00            |
| hL1-26/50-53|                 | 0.37 ± 0.00            |
| hL1-26/64-68|                 | 0.30 ± 0.00            |
| hL1-26/75-76|                 | 0.49 ± 0.00            |
| hL1-26/81 |                     | 0.62 ± 0.00            |
| hL1-81   |                     | 0.40 ± 0.00            |
| hL1-1741-81|                 | 0.74 ± 0.00            |
| hL41-81  |                     | 0.72 ± 0.00            |
| hL6/64-81|                     | 0.35 ± 0.00            |

Figs. 1 and 2 illustrate the amino acid sequences of the hormones and analogs used in this study relative to the overall structures of the α- and β-subunits. Characterization of the immunological properties of each α- and β-subunit analog has been described (1). All but two of the β-subunit analogs have also been described (20, 28). The α-subunit sequence of hCG is unique in loop 1 was not required for increased activity. Other analogs have also been described (20, 28). CLC89–96, an hLH/hCG analog has been described (1). All but two of the α-subunit chimeras were assayed using an α-subunit antibody (A113) as a capture agent and a labeled antibody (B410) that has high affinity for nearly all mammalian LH β-subunits, including those from hCG and bLH (30). Again, we used highly purified urinary hCG as a standard. We also used this same assay to quantify heterodimers consisting of human/bovine α-subunit chimeras. However, because A113 has lower affinity for the α-subunit from bLH than that from hCG (1), we used highly purified hCG as the standard in these latter assays. Procedures for preparing radiolabeled hCG and antibodies using IODO-GEN (Pierce) to specific activities of approximately 50 μCi/μg have been described (31).

RESULTS

The Small Influence of the bLH α-Subunit on Receptor Binding Is Not Sufficient to Account for the Inability of bLH to Bind bLH—Consistent with earlier reports (32), we found that the heterodimer consisting of the bovine α-subunit and hCG β-subunit bound rLHR well (Table I). This analog bound bLH about half as well as hCG (Table I), but more than 1000-fold better than bLH, indicating that differences between the human and bovine α-subunits per se were not responsible for the very low activity of bLH in hLHR assays. To localize residues for the small loss in binding to the hLHR, we compared the activities of heterodimers containing hCG β-subunit and human/bovine α-subunit chimeras (Figs. 1 and 2). Many of these were more active than those containing either the human or bovine α-subunits (Table I). While some of the most active analogs (e.g. hB11–26/64–81) contained bovine residues in loop 1 and human residues in all or parts of loop 3, the presence of bovine residues in loop 1 was not required for increased activity. Other analogs (e.g. hB11–26/64–68, bH11–26/75–79, and bH11–26/81) were more active than hCG even though they contained human residues in loop 1 and bovine residues in loop 3 (Table I). This suggested that the increased activities of some chimaeras was caused by interactions between residues in the regions of the α-subunit that differ most in the human and bovine proteins (i.e. the N terminus, loop 1, and loop 3) rather than by interactions between bovine-specific α-subunit residues and either of the receptors.

A Portion of the bLH β-Subunit in Loops Two and/or Three...
Hada Small Influence on Binding to hLHR, but Not Enough to Account for the Inability of bLH to Bind to hLHR—Heterodimers containing the human α-subunit and bLH/hCG β-subunit chimeras bound hLHR at least 100-fold better than bLH, suggesting that residues in the bLH β-subunit per se accounted for only a fraction of the low affinity of bLH for the hLHR. The design of the chimeras permitted an assessment of differences in β-subunit loop 1, loops 2–3, and the seat belt (Table II). The presence of bLH residues in loops 2–3 had a greater influence than those in loop 1 or the seat belt (Fig. 3, top, Table II, column labeled “human α-subunit”) as seen by comparing the relative activity of an analog containing bLH β-subunit residues only in loop 1 (i.e. CbL36–145) with that of an analog containing bLH β-subunit residues in loops 1–3 (i.e. CbL58–145). The influence of loops 2–3 can also be seen by comparing the activity of an analog containing bLH seat belt residues (i.e. CbL1–87) with that of an analog containing the bLH seat belt and β-subunit residues derived from loops 2–3 (i.e. CbL1–35). Heterodimers containing human α-subunit and bLH β-subunit residues only in loop 1 (i.e. CbL36–145) or the seat belt (i.e. CbL1–87) were about as active as hCG.

The Low Potency of bLH in the hLHR Binding Assay Is Due to a Combined Influence of the Bovine α-Subunit and the Seat Belt Portion of the bLH β-Subunit—To test the idea that the inactivity of bLH was due to the combined effects of bovine residues in both subunits, we assayed heterodimers that contained bovine α-subunit and portions of the bLH β-subunit in which loops 2–3 originated from bLH. Although analogs containing the bovine α-subunit and these bLH β-subunit residues were less active than those containing the human α-subunit (Table II), they were more active than bLH in hLHR assays. For example, the hLHR activities of heterodimers containing the entire bovine α-subunit and bLH β-subunit residues solely in loop 1 (CbL36–145) or loops 1–3 (CbL58–145) were reduced 50–80% relative to those that contained the human α-subunit (Fig. 3, top and bottom, and Table II), yet they remained at least 100-fold greater than bLH.

The least active chimeras contained the bovine α-subunit and the bLH seat belt. This can be seen by comparing the activities of heterodimers containing the bovine α-subunit and bovine β-subunit residues and CbL1–87, a β-subunit chimera containing hCG loops 1–3 and the bLH seat belt (Figs. 3 and 4, Table II). The heterodimer containing the human α-subunit had nearly the same potency as hCG; that containing the bovine α-subunit had nearly the same potency as hCG; that containing the bovine α-subunit was inactive at the highest concentration available for testing.

The relative influences of β-subunit loops 1–3 and the seat belt...
Binding of bLH to hLHR

This table illustrates the activities of heterodimers containing either the human or bovine α-subunit and the β-subunit analogs. All values are potencies relative to that of hCG and are the means ± S.E. of at least two experiments (n). The block diagram refers to the structures of the chimeras prepared as described in the text or in an earlier publication (20). Loop 1 refers to β-subunit residues 9–34. Loops 2 and 3 refer to β-subunit residues 35–88. SB refers to the seat belt β-subunit residues 91–110. Note, residues in the N- and C-terminal halves of the seat belt have been shown to influence the abilities of hCG to bind to LH and FSH receptors, respectively (1). NT refers to not tested. Note also, CbL103–145 refers to an analog that has the N-terminal half of the bLH seat belt and the C-terminal half of the hCG seat belt. *Denotes that the activity of the bovine α-subunit/hCGβSS5L heterodimer was estimated from the ED50 obtained in two experiments, one of which is shown in Fig. 7. This value appears to be a maximum estimate since the displacement of [125I-hCG] by the analog was not parallel to that of hCG. Much higher amounts of this analog relative to those of hCG would have been required to inhibit binding of the radiolabeled to an equivalent extent.

Identification of Seat Belt Residues That Influence Binding to hLHR—Previous studies showed that seat belt residues influence the receptor binding specificity of hCG (21). Those between Cys93 and Cys100 could lead to a reduction in hLHR binding. To test this, we compared the activities of heterodimers containing either the human or bovine α-subunit and bLH β-subunit analogs. That containing the bovine α-subunit, bLH β-subunit loops 1–3, and the hCG seat belt (i.e., CbL88–145) was at least 100-fold more active than that containing the bovine α-subunit, hCG β-subunit loops 1–3, and the bLH seat belt (i.e., CbL1–87). These observations showed that the effect of the interactions between bLH-specific residues in the α-subunit and seat belt was considerably greater than those in the α-subunit and β-subunit loops 1–3.

Table II

Summary of β-subunit mutations on the activities of heterodimers in hLHR assays

| β-Subunit Region of β-Subunit | Relative hLHR Potency (hCG = 1.00) |
|-----------------------------|----------------------------------|
|                            | human α-subunit | bovine α-subunit |
| hCG                        | 1.00 ± 0.00     | 0.57 ± 0.27(4)  |
| hLH                        | NT              | <0.001          |
| CbL1–35                    | 0.11 ± 0.00(4)  | <0.001          |
| CbL1–87                    | 0.84 ± 0.15(5)  | <0.001          |
| CbL88–145                  | 0.75 ± 0.15(4)  | 0.15 ± 0.04(2)  |
| CbL103–145                 | 0.18 ± 0.00(3)  | 0.10 ± 0.04(3)  |
| CbL90–92                   | 0.31 ± 0.00(4)  | <0.001          |
| CbL89–145                  | 1.25 ± 0.10(2)  | 0.46 ± 0.08(2)  |
| CbL90–145                  | 0.90 ± 0.16(2)  | 0.005–0.01*     |

Fig. 3. Influence of β-subunit on binding of heterodimers to hLHR. Top, heterodimers containing human α-subunit and β-subunit chimeras were produced by transient transfection of COS-7 cells with vectors encoding hCG α-subunit (21) and the chimera β-subunits (20). Their abilities to bind to hLHR were measured as described in the text as noted on the figures. The hCG and bLH, gifts from Drs. Robert Canfield, Steven Birken, and John Pierce, were purified standards obtained from urine and from the bovine anterior pituitary gland, respectively. Vertical bars extend to the limits of the S.E. Results are typical of at least three experiments. Bottom, these data are similar to those in the top panel except that all the recombinant heterodimers contained the hLH α-subunit produced by transfection of COS-7 cells with vectors encoding a synthetic bLH α-subunit gene (33) and the chimeric β-subunits (20).

activities of heterodimers containing either the human or bovine α-subunit and an hCG/hLH chimeric β-subunit (CLC89–92) containing Gly91 and Pro92. In hLHR assays, the bovine α-subunit/CLC89–92 β-subunit heterodimer was similar in activity to that containing the bovine α-subunit and the hCG β-subunit. Both had approximately half the activity of hCG (Table II). Thus, the differences at β-subunit residues 91 and 92 were not responsible for the very low ability of bLH to bind to hLHR.

Finally, we studied the role of Leu95, the residue that was the least conserved in this region of the hCG and hLH seat belts. Heterodimers containing the human α-subunit and an hCG β-subunit analog in which Arg85 was converted to Leu (i.e.,
concentrations of more than 10 for the hLHR. In principle, this would enable us to identify containing parts of lutropins that had both high and low affinities we characterized the hLHR binding activities of chimeras containing parts of lutropins that had both high and low affinities for the hLHR. In principle, this would enable us to identify clusters of amino acids that would contain residues responsible for the inactivity of bLH. Subsequent mutagenesis would need focus only on these smaller regions. This strategy has been shown to be highly efficient (35).

When these studies were initiated, we expected residues, which differed in hCG and bLH in one or both subunits, would participate in high affinity hLHR contacts and be recognized by contributions they made to the overall affinity of the hormone for the receptor. Given that bLH is virtually inactive in hLHR assays, we were surprised to find that heterodimers containing one hCG subunit were highly active. Those containing the hCG β-subunit and a chimeric α-subunit were at least half as active as hCG; some were even more active than hCG in both rLHR and hLHR assays. The activities of heterodimers containing the hCG α-subunit and bLH-specific residues in β-subunit loops 2–3 were reduced only 5–10 fold. Even when present throughout loops 1–3 of both subunits, bLH-specific residues reduced binding only 10–20 fold. Taken together, this suggests that residues that differ in hCG and bLH are probably not essential for receptor binding. In marked contrast to the effects of residues in β-subunit loops 1–3, the combined influence of a single residue (Leu95) in the bLH seat belt and the bLH α-subunit accounted for most of the inactivity of bLH in hLHR assays. Thus, while neither the entire bLH seat belt nor Leu95 had a significant influence on the hLHR binding activities of heterodimers containing the human α-subunit, the activities of heterodimers containing these residues and the bLH α-subunit were reduced 1000-fold, nearly to the level of bLH. This extreme synergism between the bLH α-subunit and the bLH seat belt suggested that differences in the abilities of hCG and bLH to bind hLHR are due primarily to an influence of the seat belt on subunit interaction.

These observations have important implications for efforts to identify residues responsible for high affinity glycoprotein-hormone receptor interactions. Because there is no crystal structure of any glycoprotein hormone-receptor complex, most efforts to decipher portions of the hormone that might interact with the receptor are based on monitoring changes in receptor binding or function following chemical or enzymatic modification (7), site-directed mutagenesis (13–19), chemical cross-linking (36–38), or competition with hormone fragments (8–12). Due to the unusually high proportion of residues in the subunit
interface (39), many mutations distant from the receptor contact sites may influence subunit interaction and thereby alter receptor binding and/or signal transduction. This would account for the observations noted earlier that some parts of the hormones previously suggested to make important receptor contacts appear to be exposed in the hormone-receptor complex or can be deleted without disrupting hormone activity. The influence of small changes in gonadotropin conformation on receptor binding is readily explained by the model in which the hormone binds to the concave surface of a horseshoe-shaped receptor extracellular domain and contacts it at two distinct sites (1). A small change in the conformation of a heterodimer that interferes with either of these contacts would be expected to reduce binding in a fashion similar to that observed between the interaction between the bovine α-subunit and the bLH seat belt.

Acknowledgments—We thank the following individuals for reagents used in these studies: Drs. Robert Canfield and Steven Birken for purified urinary hCG and antibodies B105, B109, and B201; Dr. John Pierce for purified bLH; Drs. Glenn Armstrong and Robert Wolfert for antibodies A113 and B112; Dr. Janet Roser for the universal anti-lutropin antibody; and Dr. Aaron Hsueh for the hLHR cDNA.

Note Added in Proof—While this manuscript was in review, Szkudlinski et al. (40) reported that the presence of bovine α-subunit residues in loop 1 increased the potency of hCG similar to what we report in Table I. However, they reached a different conclusion, namely that these residues contact the receptor as predicted by the model of Jiang et al. (2).

REFERENCES

1. Moyle, W. R., Campbell, R. K., Rao, S. N. V., Ayad, N. G., Bernard, M. P., Han, Y., and Wang, Y. (1995) J. Biol. Chem. 270, 20020–20031
2. Jiang, X., Dreano, M., Buckler, D. R., Cheng, S., Ythier, A., Wu, H., Hendrickson, W. A., and Tayar, N. E. (1995) Structure (Lond.) 3, 1341–1353
3. Laphorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) Nature 369, 455–461
4. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) Structure (Lond.) 2, 545–558
5. Kobe, B., and Deisenhofer, J. (1993) Nature 366, 751–756
6. Kobe, B., and Deisenhofer, J. (1995) Nature 374, 183–186
7. Pierce, J. G., and Parsons, T. F. (1981) Annu. Rev. Biochem. 50, 465–495
8. Keutmann, H. T., Charlesworth, M. C., Mason, K. A., Ostrea, T., Johnson, L., and Ryan, R. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2038–2042
9. Keutmann, H. T., Mason, K. A., Kitzmann, K., and Ryan, R. J. (1989) Mol. Endocrinol. 3, 526–531
10. Reed, D. K., Ryan, R. J., and McCormick, D. J. (1991) J. Biol. Chem. 266, 14251–14255
11. Roche, P. C., Ryan, R. J., and McCormick, D. J. (1992) Endocrinology 131, 268–274
12. Coloma, T. A., Dattatreyamurty, B., and Reichert, L. E., Jr. (1990) Biochemistry 29, 1104–1109
13. Chen, F., Wang, Y., and Puetz, D. (1991) J. Biol. Chem. 266, 19357–19361
14. Chen, F., and Puetz, D. (1991) Biochemistry 30, 10171–10175
15. Xia, H., Huang, J., T., M., and Puetz, D. (1993) J. Mol. Endocrinol. 10, 337–343
16. Yao, J., Ji, I., and Ji, T. H. (1991) J. Biol. Chem. 266, 17741–17743
17. Yao, J., Zheng, H., Ji, I., Murdoch, W., and Ji, T. H. (1993) J. Biol. Chem. 268, 13034–13042
18. Liu, C., Roth, K. E., Lindau Shepard, B. A., Shaffer, J. B., and Dias, J. A. (1993) J. Biol. Chem. 268, 21613–21617
19. Lindau-Shepard, B., Roth, K. E., and Dias, J. A. (1994) Endocrinology 135, 1235–1240
20. Cosowsky, L., Rao, S. N. V., Macdonald, G. J., Papkoff, H., Campbell, R. K., and Moyle, W. R. (1995) J. Biol. Chem. 270, 20911–20919
21. Campbell, R. K., Dean Emig, D. M., and Moyle, W. R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 760–764
22. Moyle, W. R., Campbell, R. K., Myers, R. V., Bernard, M. P., Han, Y., and Wang, X. (1994) Nature 368, 251–255
23. Blithe, D. L., Richards, R. G., and Skarulis, M. C. (1991) Endocrinology 129, 2237–2259
24. Jia, X.-C., Oikawa, M., Be, M., Tanaka, T., Ny, T., Boime, I., and Hsueh, A. J. W. (1991) Mol. Endocrinol. 5, 759–768
25. Davies, T. F., Walsh, P. C., Hodgen, G. D., Dufau, M. L., and Catt, K. J. (1979) J. Clin. Endocrinol. & Metab. 48, 680–685
26. Southern, P., and Berg, P. (1982) J. Mol. Appl. Genet. 1, 327–341
27. Bernard, M. P., Myers, R. V., and Moyle, W. R. (1990) Mol. Cell. Endocrinol. 71, R19–R23
28. Moyle, W. R., Matzuk, M. M., Campbell, R. K., Cogliani, E., Dean-Emig, D. M., Krichevsky, A., Barnett, R. W., and Boime I. (1990) J. Biol. Chem. 265, 8511–8518
29. Moyle, W. R., Ehrlich, P. H., and Canfield, R. E. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2245–2249
30. Matteri, R. L., Roser, J. F., Baldwin, D. M., Lipovetsky, V., and Papkoff, H. (1987) Domest. Anim. Endocrinol. 4, 157–165
31. Cruz, R. I., Anderson, D. M., Armstrong, E. G., and Moyle, W. R. (1987) J. Clin. Endocrinol. & Metab. 64, 433–440
32. El Deiry, S., Chen, T. M., and Puetz, D. (1991) Mol. Cell. Endocrinol. 76, 105–113
33. Campbell, R. K., Erle, H., Barnett, R. W., and Moyle, W. R. (1992) Mol. Cell. Endocrinol. 83, 195–200
34. Chen, F., Wang, Y., and Puetz, D. (1992) Mol. Endocrinol. 6, 914–919
35. Wells, J. A. (1991) Methods Enzymol. 202, 390–394
36. Keutmann, H. T., and Rubin, D. A. (1993) Endocrinology 132, 1305–1312
37. Ji, I., and Ji, T. H. (1990) Proc. Natl. Acad. Sci. U. S. A 87, 4306–4400
38. Weare, J. A., and Reichert, L. E., Jr. (1979) J. Biol. Chem. 254, 6972–6979
39. Jones, S., and Thornton, J. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 93, 13–20
40. Szkudlinski, M. W., Teh, N. G., Grossmann, M., Tropea, J. E., and Weintraub, B. (1994) Nat. Biotechnol. 14, 1257–1263