Endocrine, *vol. 20, no. 1, 177–189, February 2003* 0969–711X/03/20:177–189/$20.00

New Homologous Bioassays for Human Lactogens Show That Agonism or Antagonism of Various Analogs Is a Function of Assay Sensitivity

Sophie Bernichtein, Sébastien Jeay, Roland Vaudry, Paul A. Kelly, and Vincent Goffin

INSERM Unit 584, Hormone Targets, Faculty of Medicine Necker, Paris Cedex, France

The reference bioassay for lactogens is the Nb2 cell proliferation assay, whose extreme sensitivity allows the detection of very low amounts of lactogenic activity in biologic fluids. The use of rat Nb2 cells raises the problem of species specificity when analyzing lactogens of other origin, including human lactogenic hormones for which no reference bioassay currently exists. In this article, we describe two new homologous bioassays for human lactogens. One is a transcriptional bioassay generated by stably transfecting 293 human embryonic kidney fibroblasts using two plasmids, encoding the human prolactin receptor (hPRLR) and the PRL-responsive lactogenic hormone response element–luciferase reporter gene. The second is a proliferation assay obtained by stably transfecting Ba/F3 cells with a plasmid encoding the hPRLR. We provide characterization of the various clones or cell populations that were isolated, and we describe experiments that were performed to achieve optimized protocols for both bioassays. These new assays were compared with other cells types exhibiting well-recognized PRL-mediated responses (proliferation of Nb2 or of human breast tumor cell lines), using various lactogen analogs. This comparative analysis provides strong evidence that the intrinsic characteristics of each bioassay dramatically affect the biologic properties attributed to the lactogen of interest. Depending on the assay, a given analog can exhibit agonistic or antagonistic properties. We hypothesize that in addition to species specificity, assay sensitivity is the key parameter in directing the apparent bioactivity of lactogens. Of course, in the end, it will be necessary to confirm the agonistic or antagonistic properties of the tested analogs, in vivo.

Key Words: Prolactin; lactogen receptor; bioassay; agonism; antagonism; Nb2 cells.

Introduction

Proliferation of rat lymphoma Nb2-11C cells has been the reference bioassay for lactogens for the last 20 yr *(1,2)*. These pre-T-lymphocytes are totally dependent on lactogenic hormones (or interleukin-2 [IL-2]) for growth; therefore, the rate of cell division can be used to quantify the amount of lactogens present in biologic fluids such as blood or culture supernatant. This bioassay has also been used extensively for structure-function studies aimed at evaluating the effect of mutations on the lactogenic activity of various hormones *(3–5)*. Moreover, these lactogen-responsive pre-T-lymphocytes were also used as a model to investigate several aspects of lactogen receptor–mediated intracellular signaling in immune cells, which has to date generated a huge body of literature *(6)*. Use of the Nb2 proliferation assay presents several advantages. First, as the most popular bioassay for lactogenic hormones, using these cells allows separate laboratories working in the same field to directly compare their results (e.g., for studies involving structure-function analysis of the same hormone). Second, since maximal proliferation is achieved at ~1 ng/mL (50 p*M*) of lactogen *(1)*, detection of lactogens as low as 10 pg/mL can be reliably performed *(2)*. Third, the rate of cell division is high (cell cycle of ~20 h in the presence of serum) so that the amplitude of the biologic response is not difficult to monitor. Fourth, cell division is among the easiest biologic responses to be quantified, and experimental data reported to date did not appear to depend on the methodology used (cell counting, ^{3}H-thymidine incorporation, colorimetric [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide] MTT-like assays).

Despite these characteristics, the Nb2 cell proliferation assay is not always totally satisfactory. First, the origin of Nb2 cells (rat) raises the question of species specificity when using ligands from other species *(7)*. Second, because of their extreme sensitivity to low amounts of lactogens, these cells are not appropriate for evaluating the antagonistic properties of mutated ligands. Third, our group showed 10 yr ago that the lactogen receptor expressed in these cells is a mutated isoform of the classical prolactin receptor (PRLR) *(8)*, distinct from the short and long receptor isoforms naturally found in the rat *(9)*. The Nb2 isoform, also referred to as "intermediate" form, lacks 198 amino acids in the cyto-

Received November 7, 2002; Accepted December 4, 2002.

Author to whom all correspondence and reprint requests should be addressed: Dr. Vincent Goffin, INSERM Unit 344, Endocrinologie Moléculaire, 156, rue de Vaugirard, 75730, Paris Cedex 15, France. E-mail: goffin@necker.fr

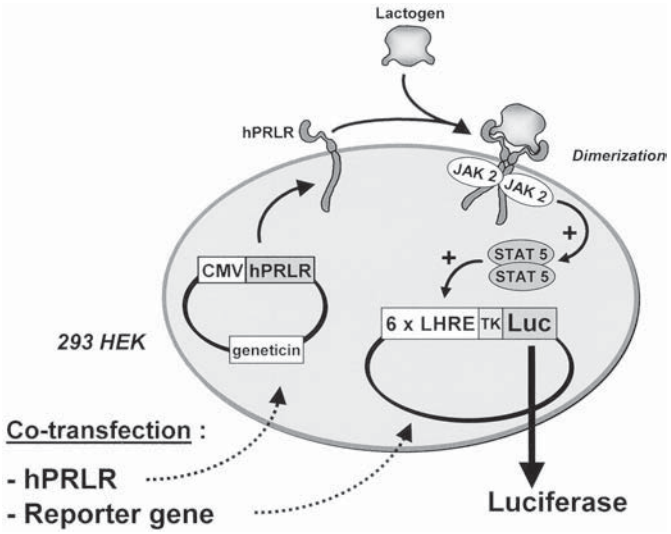


Fig. 1. Human PRLR-mediated transcriptional bioassay (HL clones). HEK 293 fibroblasts were cotransfected with two plasmids encoding the hPRLR and a PRL-responsive luciferase (Luc) reporter gene including as the promoter a six-repeat sequence of the LHRE (which is the DNA-binding element of STAT5). When 1:2 active complexes are formed (one ligand, two receptors), the receptor-associated tyrosine kinase Jak2 is activated and in turn activates the transcription factor STAT5, which results in transcriptional activation of the reporter gene. Thus, the luciferase activity reflects receptor activation by the exogenous PRLR ligand, i.e., formation of active 1:2 complexes.

plasmic domain (residues 323–520 in the long isoform) *(8)*, and although this receptor is considered to exhibit signaling properties similar to the long isoform *(10)*, the consequence of the deletion on biologic responses exhibited by Nb2 cells has not been thoroughly studied.

For the past 12 yr, our group has been interested in deciphering the interaction between human PRL (hPRL) and the lactogen receptor *(11)*. In 1994, we concluded from Nb2 assay experiments that the hPRL mutant in which an arginine was substituted for the helix 3 glycine (G129R analog) was not an antagonist, in contrast to our expectations *(12)*. In 1995, Dattani et al. *(13)* reported that the human growth hormone (hGH) variant carrying the homologous glycine mutation (G120R analog) had versatile, zinc-dependent agonistic activity on Nb2 cell proliferation, whereas the original report showed that G120R-hGH antagonizes proliferation and had, at best, only minimal agonistic activity *(14)*. These apparently contradictory observations regarding the molecular bases of lactogen receptor antagonism prompted us to develop a homologous bioassay for human lactogens. Human embryonic kidney fibroblasts (293 HEK) were transiently transfected using two plasmids encoding the long isoform of the hPRLR *(15)*, and a PRL-responsive luciferase reporter gene *(7)* (Fig. 1). Using this bioassay, we were able to demonstrate, first, the species specificity of some characteristics of the hormone-receptor interaction (rat vs human receptor), and, second, that G129R-hPRL analog is a potent antagonist of the human lactogen receptor *(7)*.

There is currently no standardized homologous bioassay for human lactogens. Although some human breast tumor cell lines express high levels of PRLR (T-47D, MCF-7), they proliferate only moderately in response to lactogen stimulation (1.5 to 4-fold over 3 to 4 d) *(16,17)*. Moreover, these cells are known to rapidly evolve with increasing passage number *(18)*, which prevents their use as an interlaboratory reference bioassay for lactogens. Thus, the present work was aimed at developing hPRLR-mediated bioassays to be used for structure-function studies of human lactogen analogs. The first bioassay described is a stable clone version of the luciferase bioassay initially developed as a transient transfection assay (Fig. 1). We also generated a population of pro-B Ba/F3 cells stably expressing the long isoform of the hPRLR, which proliferate in response to PRL stimulation. This assay is similar to the Nb2 cell assay, with the difference that the biologic response is mediated by the human receptor. Not only have these new bioassays been shown to be appropriate tools for characterizing human lactogens, but, more important, they have greatly helped in understanding why a given lactogen analog exhibits agonistic properties in one bioassay and is an antagonist in another.

Results

Transcriptional Assay (Clone HL5)

Clone Selection and Assay Conditions

The principle of the transcriptional assay is to monitor the ability of lactogens to activate the hPRLR by measuring the level of STAT5-mediated transcriptional activation of a PRL-responsive reporter gene, containing a six-repeat lactogenic hormone response elements (LHRE) from the β-casein promoter (Fig. 1). After transfection and geneticin selection of stable 293 HEK clones, 32 stable clones were screened for their luciferase response in the presence of 1 μg/mL of hPRL. Three clones (named HL5, HL10, and HL21, "HL" for Human PRLR-Luciferase) were selected based on their ≥10-fold induction of luciferase activity after 24 h of hormone treatment. After preliminary screening, dose-response curves were performed using these selected clones, which revealed different sensitivity to PRL stimulus, with >90% luciferase response obtained at 1 μg/mL of hPRL for clones HL5 and HL21, and only at 100–200 ng/mL for clone HL10 (Fig. 2A). Moreover, relative light units (RLU) also strongly differed, with values within the hundred to low thousand range for clone HL10, and within the low to hundred thousand range for clones HL5 and HL21 (Fig. 2A).

Using clone HL5 and recombinant hPRL as the ligand, various experimental conditions were then tested to set up the optimized protocol, including serum concentration in the assay medium (0–10%), appropriateness and duration of serum starvation (0–24 h), cell density (up to 10^5 cells/well of 96-well plates), and duration of PRL stimulation (24–48

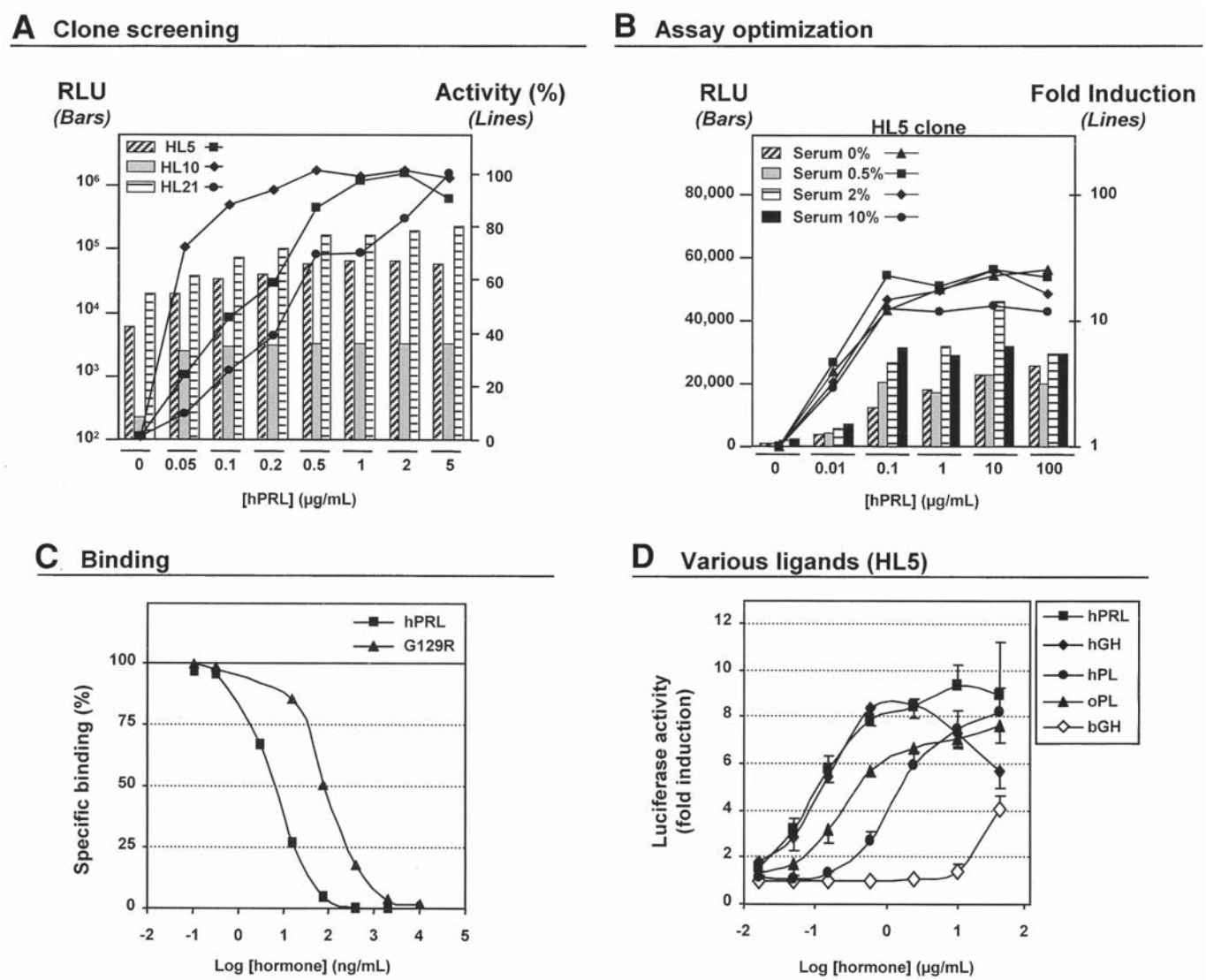


Fig. 2. Optimization and characterization of transcriptional bioassay. (**A**) Clones HL5, HL10, and HL21 were screened for their luciferase response in the presence of increasing amounts of hPRL. Values are expressed in RLU (bars, left axis) and percentage of maximal activity (lines, right axis); see text for comments. (**B**) Dose response of hPRL on HL5 clone was assayed using medium containing increasing amounts of serum. Values are expressed in relative light units (bars, left axis) and fold induction (lines, right axis). High serum concentrations reduced fold induction owing to higher RLU values in nonstimulated cells (see text). (**C**) Typical competition curves between [^{125}I]-hPRL and unlabeled hPRL or G129R-hPRL for binding to the hPRLR obtained from HL5 cell lysates. (**D**) Various proteins of the PRL/PL/GH family were tested for their ability to stimulate HL5 cell luciferase response. All lactogens tested activated the hPRLR, whereas nonlactogenic bovine hGH had a partial effect only at an extreme concentration.

h). Basically, the higher the serum concentration the higher the absolute RLU values (Fig. 2B), although background values were more affected than PRL-stimulated luciferase response, indicating the presence in serum of factors activating STAT5 (lactogens or other cytokines/growth factors). Hence, we selected the assay medium containing 0.25–0.5% serum, which was sufficient to maintain maximal luciferase fold induction without preventing cell adhesion in 96-well plates. An average of 50,000 cells/well appeared to be optimal, and we did not observe any advantage in prolonging the assay over 24 h. We also ensured that PRL did not stimulate proliferation of HL5 cells (not shown), which might have interfered with the amplitude of the transcriptional response measured.

The protocol selected from these trials was as follows: Fifty to 90% confluent cells are trypsinized, centrifuged, and resuspended at a density of 500,000 cells/mL in 0.5% serum medium. Cells are distributed in 96-well plates (100 µL/well) and allowed to adhere for 6–18 h (overnight), and then 100 µL of twofold concentrated hormones diluted in

serum-free medium are added for an additional 24-h period. Cells are then lysed in 50 μL of lysis buffer and luciferase activity is monitored using 10–20 μL of lysates as indicated in Materials and Methods. We routinely observed that RLU values decreased with cell passages, without strongly affecting the amplitude (fold induction) of the biological response, which was always about 10-fold or more. To circumvent this problem, we routinely analyzed all lactogen analogs to be compared in the same assay.

Characterization of Clone HL5

While parental 293 HEK cells express undetectable amounts of lactogen receptors, Scatchard analysis performed using cell lysates indicated that clone HL5 expresses 63,000 receptors/cell, with an affinity for hPRL in the expected range (K_d of $3.4 \pm 1.3 \times 10^{-10}$ *M*) (Fig. 1C). The competition curve of G129R-hPRL was shifted to the right by 1 log unit, indicating a 10-fold lower affinity *(19)*.

Several dose-response experiments were performed to evaluate the sensitivity of clone HL5. Concentrations <10 ng/mL could hardly be detected. More than 90% of maximal activity was routinely achieved at 1 to 2 μg/mL of hPRL, although in some experiments, the curve further increased up to 10 μg/mL. Dose-response curves along a wide range of concentrations appeared to exhibit a bell shape, reflecting hormone self-antagonism at high concentrations (>50 μg/mL). In addition, various hormones of the PRL/GH/PL (placental lactogen) family (hPRL, hGH, hPL, ovine PL [oPL], and bovine GH [bGH]) were tested to ensure specificity of the luciferase response (Fig. 2D). Expectedly, all lactogens activated the PRL-responsive reporter gene, although with some different abilities. Relative activities of hPRL and hGH were almost identical, and superior to that of oPL and hPL. bGH slightly activated the reporter system at very high concentrations (>10 μg/mL). Since we used recombinant hormone, the presence of lactogen contaminants in the bGH preparations is unlikely. Expression of low amounts of somatogen receptors in 293 HEK cells (unpublished observation) is a possible explanation since the GH receptor also activates the Jak2-Stat5 cascade *(20)*. However, the small effect of pure somatogens in this bioassay is negligible since the dose-response curve of bGH is displaced toward the high concentrations by almost 2 log units compared to the less potent lactogen in the assay, placental lactogen.

Proliferation Assay (Ba/F3-LP and HP)

Cell Selection and Assay Conditions

Parental Ba/F3 cells were transfected using a plasmid encoding the hPRLR, and then cells stably expressing the receptor were geneticin selected. Substitution of recombinant hPRL (1 μg/mL) for IL-3 further ensured selecting cells PRL dependent for their growth. After a few passages in these conditions, the stable population was split into three subpopulations routinely passaged in culture medium containing 10, 100, or 1000 ng/mL of hPRL. These subpopulations were further referred to as LP (low PRL), MP (medium PRL), and HP (high PRL), respectively. Preliminary proliferation assays indicated that dose-response curves peaked at 10 ng/mL for LP cells, whereas MP and HP cells had maximal proliferation at ~100 ng/mL. Optimization of assay conditions was performed using LP and HP populations. Basically, we analyzed four major parameters: composition of assay medium, appropriateness and features of serum starvation, cell density, and duration of the assay.

Ba/F3 cells barely support serum starvation and rapidly undergo apoptosis *(21)*. Moreover, the high sensitivity of LP cells to low concentrations of PRL (10 ng/mL) strongly suggested that the presence of serum in the assay medium may influence cell responsiveness owing to the presence of lactogen in serum. Therefore, we monitored cell division and apoptosis under various experimental conditions using fluorescence-activated cell sorter (FACS) methodology. As shown in Fig. 3, almost 100% of the cells underwent apoptosis when the assay was performed for 3 d in the absence of serum (left, hatched bars), and PRL stimulation had only a partial protective effect against programed cell death (left, gray bars). Accordingly, the rate of cell proliferation was relatively low, especially when serum starvation lasted 24 h. By contrast, performing the assay in 1% fetal calf serum (FCS) (Fig. 3, right) appeared much less drastic regarding apoptosis in nonstimulated cells, even after 3 d. A starvation period of 6 h appeared to be the best compromise to ensure a low rate of apoptosis in unstimulated cells and a high rate of cell division in PRL-stimulated cells. A cell density of 50,000 cells/well (200 μL final) appeared to be optimal with respect to these parameters (not shown). Using higher cell density led to overcrowded wells, resulting in increased cell death after 3 d of stimulation. Otherwise, using a lower cell density limited detection of differences between background and stimulated cells (at least using WST-1 colorimetric reagent). Similarly, cell proliferation was routinely monitored after 3 d of treatment, since shorter times limited detection of differences in cell densities, and longer times increased cell death.

The protocol selected from these trials was as follows: Cells were starved for 6 h in 1% FCS medium, and then 5×10^4 cells (in 100 μL) were distributed in each well in the same medium. One hundred microliters of (2X) hormones diluted in 1% FCS medium was added per well, and cell viability was estimated after 3 d of hormonal stimulation by adding 10 μL of WST-1 tetrazolium salt. Note that the assay reflects both mitogenic and antiapoptotic activities of lactogens on these cells (Fig. 3).

Characterization of Ba/F3-LP Cells

Parental Ba/F3 cells do not express the PRLR and fail to respond to PRL. Although hPRL obviously exerts mitogenic activity on Ba/F3-LP cells, it was extremely difficult to detect specific binding to the lactogen receptor ectopically expressed by these cells. One successful Scatchard analy-

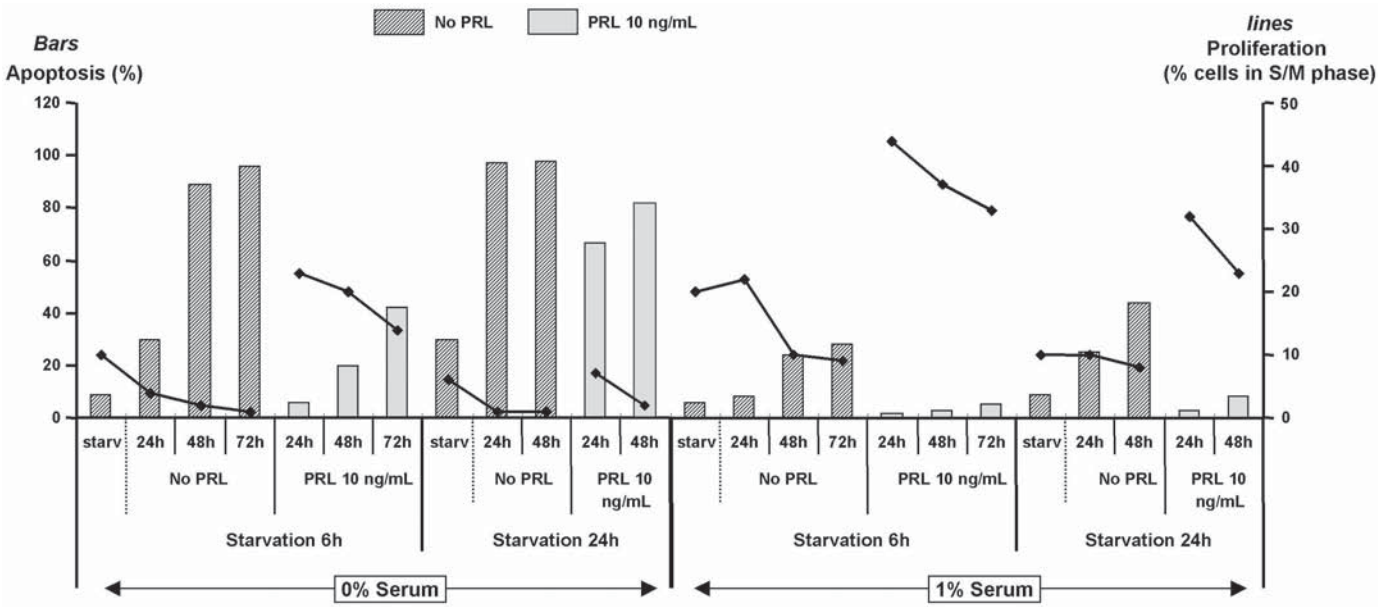


Fig. 3. Optimization of hPRLR-mediated proliferation bioassay (Ba/F3-LP). Proliferation assays using Ba/F3-LP cells were performed under various experimental conditions. Basically, we used assay medium containing no serum or 1% FCS, and we performed a starvation period in the same medium for 6 or 24 h before stimulating cells with 10 ng/mL of hPRL. The relative percentage of apoptotic (bars, left axis) vs dividing (lines, right axis) cells was quantified by FACS analysis following the starvation period (starv) and every 24 h for the next 3 d. In the absence of serum, nearly all cells underwent apoptosis in the absence of PRL stimulation; therefore, this condition was unsuitable to be used in a 3-d bioassay. In the presence of 1% serum, the level of apoptosis in the absence of PRL was much lower, while stimulated cells readily proliferated.

sis (not shown) revealed that Ba/F3-LP cells express <300 receptors/cell, with the expected affinity for hPRL (K_d = $2.5 \pm 0.9 \times 10^{-10}$ *M*). The reason for such a low expression of PRLRs in this stably transfected population is unknown. Because of the poor crossreaction of U5/U6 anti-PRLR monoclonal antibodies with the human receptor (they were raised against the rat receptor), no attempt was made to detect the receptor protein by immunoblot.

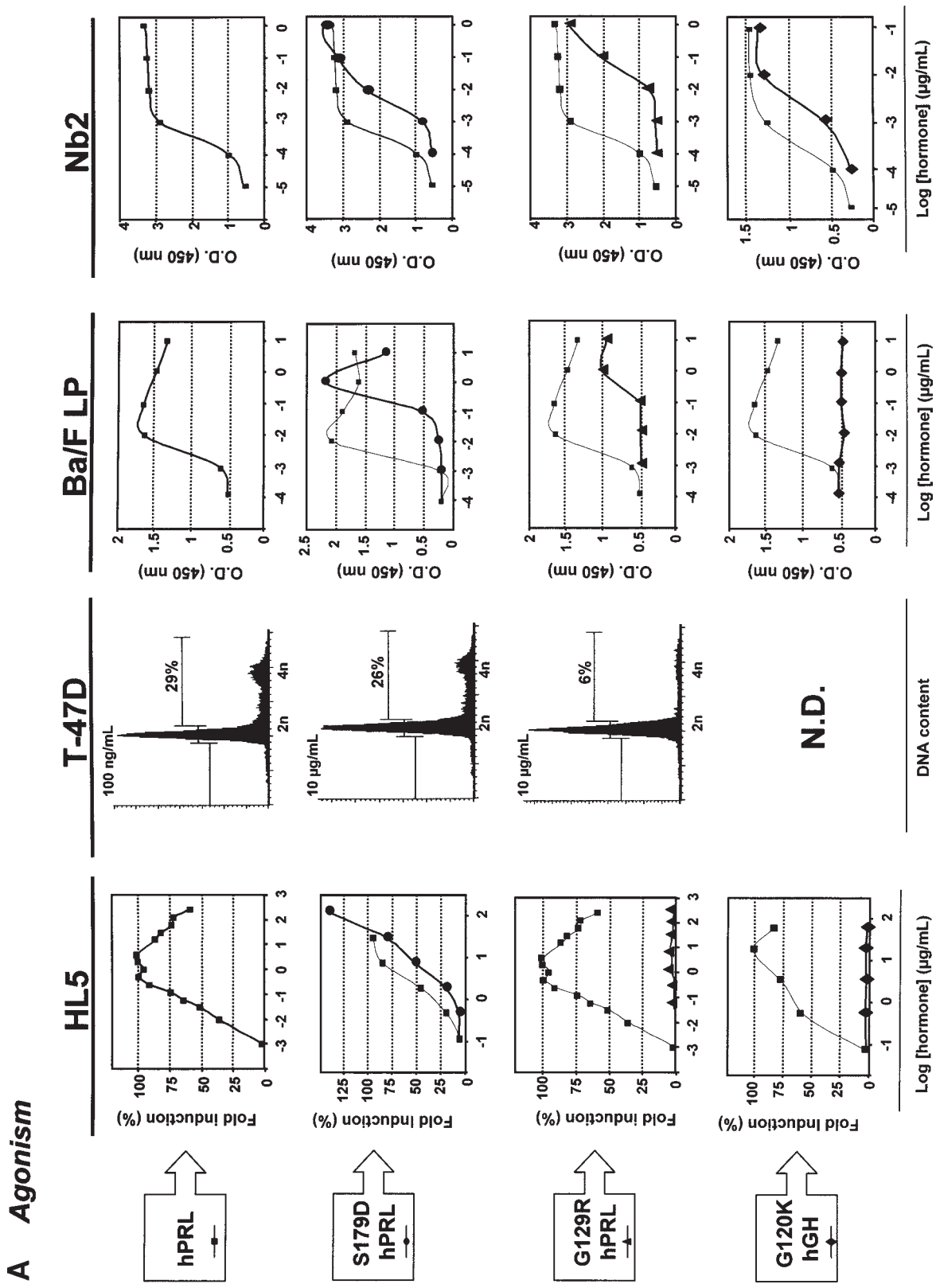
Use of New Lactogen Bioassays (Ba/F3-LP and HL5)

Within the last 3 yr, these new homologous bioassays were routinely used by our group in comparison to or in place of the reference Nb2 proliferation assay to characterize the lactogen receptor–mediated activity of several lactogen analogs. Basically, the assays were used to better characterize the agonistic vs antagonistic properties of our classical hPRL analog named G129R-hPRL, of the molecular mimicry of phosphorylated hPRL referred to as S179D-hPRL, and of hGH analogs displaying antagonistic activity such as the classic G120K analog, or its improved version B2036 (also known as Pegvisomant in its polyethylene glycol [PEG]-linked formulation). In addition, the two new assays were used in an international collaborative study aimed at characterizing the lactogenic bioactivity of a recombinant DNA-derived hPRL preparation produced in murine C127 cells *(22)*, which, along with its glycosylated and nonglycosylated components, were recently established as World Health Organization (WHO) reference reagents (unpublished).

Structure-Function Studies

Although some of the data presented next have already been published elsewhere *(19,23–25)*, the goal of this section is to provide evidence that a given lactogen analog can exhibit very different biologic properties depending on the bioassay that is used for its characterization. Expectedly, hPRL is active in the four bioassays used (Fig. 4A, top), although maximal activity is achieved at different concentrations: ~1 µg/mL (HL5) *(19)*, ~100–250 ng/mL (T-47D) *(25)*, ~10 ng/mL (Ba/F3-LP), and ~1 ng/mL (Nb2) *(3)*. In the two first assays cited, dose-response curves are routinely bell shaped, whereas this is much less pronounced using Ba/F3-LP cells and almost never using Nb2 cells.

S179D-hPRL has been reported to be a hPRL antagonist on Nb2 cell proliferation *(26)*. In our hands, this analog is clearly an agonist, not only on Nb2 cell proliferation *(23)*, but also in the three other bioassays used in the present study (Fig. 4A). In addition, its maximal bioactivity in the luciferase assay significantly exceeded that of wild-type (WT) hPRL *(23)*. In all the cases, however, dose-response curves were more or less displaced to the right (although not as much as G129R-hPRL), indicating that this lower-affinity analog requires being present in larger amounts than WT


A Agonism
HL5
T-47D
Ba/F LP
Nb2
hPRL
S179D hPRL
G129R hPRL
G120K hGH
Fold Induction (%)
O.D. (450 nm)
100 ng/mL
10 µg/mL
29%
26%
6%
N.D.
2n
4n
DNA content
Log [hormone] (µg/mL)

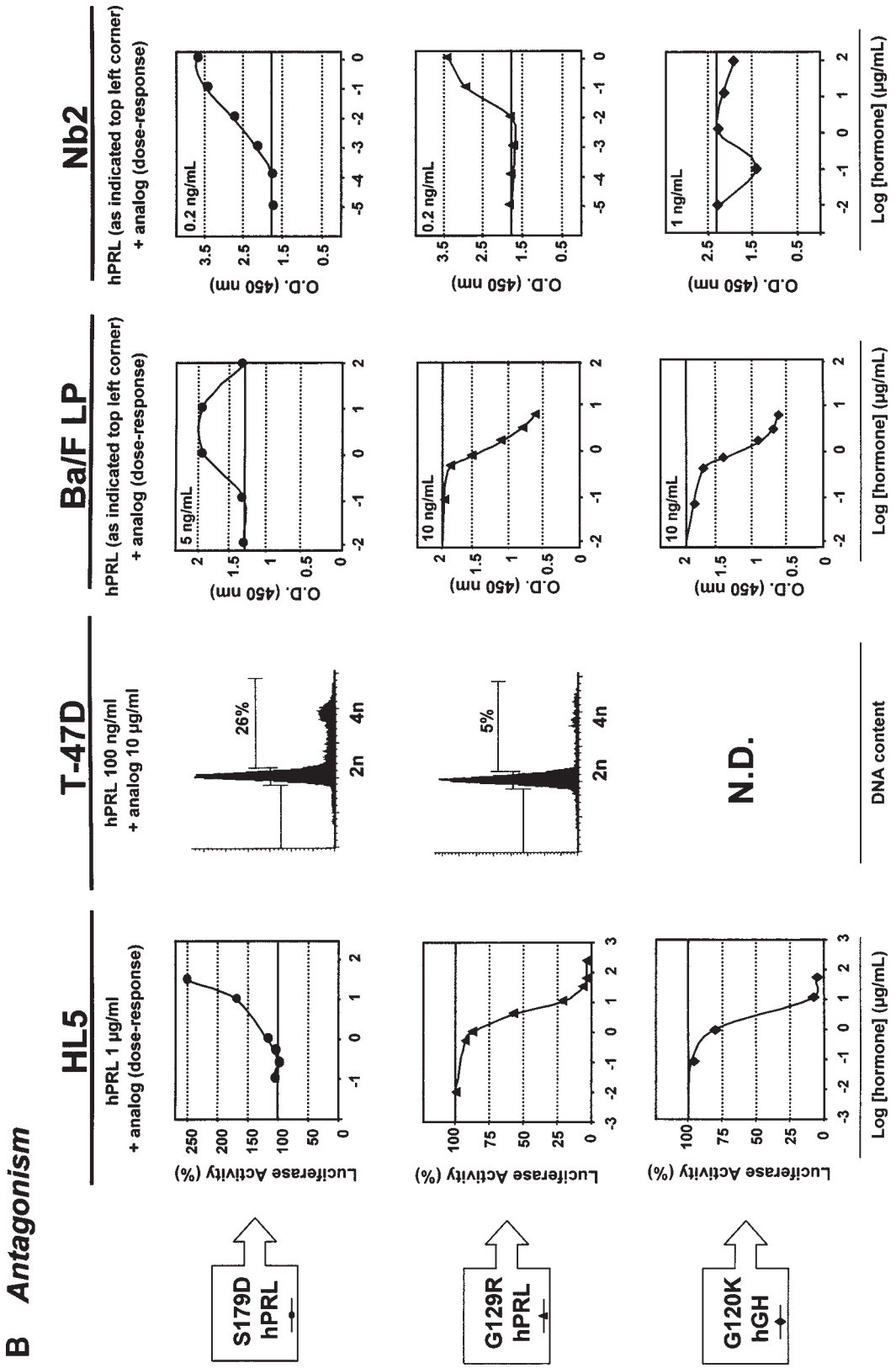


Fig. 4. Comparison of agonistic and antagonistic properties of various PRLR ligands using four different bioassays. (**A**) Agonism. WT hPRL, S179D-hPRL, G129R-hPRL, and G120K-hGH were tested for their ability to activate the PRLR using four cell lines: clone HL5 (luciferase response), T-47D (proliferation monitored by FACS), Ba/F3-LP, and Nb2 cells (proliferation monitored using WST-1 colorimetric assay). (**B**) Antagonism. Antagonistic properties of the three analogs were assessed based on their ability to compete with hPRL for activating luciferase or proliferation responses. See text for interpretation of the various panels. Note that the curves presented in individual panels were obtained within the same experiment; therefore, direct comparison of optical density (OD) values can be made to evaluate relative potency of analogs compared to WT hPRL. By contrast, absolute OD may differ from one panel to another depending on the duration of the colorimetric reaction; thus, interpanel comparison of absolute OD values has no relevance.

hPRL to achieve the same level of activity. In agreement with its agonistic activity, S179D-hPRL failed to antagonize hPRL in any of our bioassays, as highlighted by the additive effect observed when this analog was assayed together with concentrations of hPRL leading to half-maximal activity (e.g., 5 ng/mL in Ba/F-LP assay or 0.2 ng/mL in Nb2 assay; see Fig. 4B). Interestingly, a slight decrease in Ba/F-LP cell proliferation was observed at extremely high S179D-hPRL concentration (>10 μg/mL), which appeared to be even more pronounced when hPRL concentrations leading to maximal activity (e.g., 10 ng/mL or more) were used in competition experiments (data not shown). Since S179D-hPRL is intrinsically an agonist exhibiting a bell-shaped curve when assayed alone (see Fig. 4A), the slope down in antagonistic experiments (Fig. 4B and data not shown) most likely reflects self-antagonism of S179D-hPRL rather than true antagonism, as previously discussed *(23)*.

G129R-hPRL displays no significant agonistic activity in the HL5 assay (<2% vs hPRL; see Fig. 4A) *(19)*, and in agreement with its ability to bind the hPRLR, it is a potent antagonist of hPRL in the same assay (Fig. 4B). Although FACS analysis failed to display any mitogenic activity of this analog on T-47D human mammary tumor cells (Fig. 4A), we showed that a low level of activity (15% of hPRL) can be detected using WST-1 colorimetric assay *(25)*. Using both methods, its antagonistic properties are clearly displayed (Fig. 4B). In the Ba/F3-LP proliferation assay, G129R-hPRL achieved ~50% activity of hPRL (Fig. 4A), and acted as a mild antagonist, since a 100-fold molar excess was necessary to inhibit hPRL mitogenic effect (Fig. 4B). Because of its intrinsic partial agonism in this assay, it is likely that the antagonism curve obtained using G129R-hPRL reflects both true antagonism against WT hPRL (in the first part of the slope down) and self-antagonism at higher concentrations (in the second part of the slope down). Finally, G129R-hPRL achieved full activity in the Nb2 assay when added in sufficient amounts (Fig. 4A) and, consequently, failed to display any antagonistic property (Fig. 4B). Thus, this analog will be considered a pure antagonist or a mild agonist depending on the bioassay that is used.

Finally, G120R-hGH analog has been reported to be a potent antagonist toward both the somatogenic and lactogenic receptors *(14,27)*. Using HL5 and Ba/F3-LP cells, G120K-hGH (assumed to be equivalent to G120R-hGH) failed to exhibit any agonistic activity and antagonized WT hPRL. In the Nb2 assay, however, its ability to activate the PRLR is demonstrated by a clear proliferation response shifted to the high concentrations with respect to hPRL. Hence, this analog failed to significantly antagonize the WT hormone, although the dose-response curve observed in antagonism experiments appeared ambiguous as we previously reported for the PEG-linked formulation of G120K *(24)*. Although G120K-hGH and S179D-hPRL exhibit almost identical curves in the Nb2 agonistic assay, this is untrue with respect to the Ba/F3-LP assay, despite the fact that both bioassays share similar features in terms of sensitivity and biologic response (cell proliferation). Although the reason for this observation is unclear, it may be linked to species-specific properties of the PRLR (rat or human), as previously observed for lactogens *(7)*.

Analysis of WHO Reference Reagent for Recombinant hPRL

The current hPRL international standard National Institute for Biological Standards and Control (NIBSC code no. 84/500) is derived from purified hPRL extracted from human pituitaries. The newly established WHO reference reagent for hPRL, which may eventually replace the international standard, was obtained from recombinant sources (see ref. *22* for details), and the ampouled product (NIBSC code no. 97/714) was recently characterized in an extensive collaborative study involving 15 laboratories, organized by NIBSC under the auspices of WHO (unpublished). We received eight coded samples to be tested blind for their lactogen receptor–mediated bioactivity, which was performed using the Nb2, Ba/F3-LP, and HL5 assays. Three of the samples analyzed are presented in Fig. 5 as examples. Averaged from three independent experiments, samples D and H appeared very similar in terms of dose-response (ED_{50}) and maximal activity using both HL5 and Ba/F3-LP cells. Noteworthy that maximal activity of these samples was similar to that obtained using in-house recombinant hPRL (produced in *Escherichia coli*). Sample Q exhibited very different properties. Using clone HL5, the dose-response curve was displaced to the left compared to samples D and H, and maximal amplitude of the response achieved twice that of the latter. By contrast, using Ba/F3-LP cells, sample Q achieved similar maximal activity but the dose-response curve was displaced to the right compared to D/H preparations. Samples D and H turned out to contain a single hPRL preparation (code no. 97/714), whereas sample Q contained purified glycosylated hPRL (code no. 98/580). These experiments show that using our two homologous bioassays allowed us to identify two coded samples containing the same hormone and, in contrast, to distinguish one sample containing a subfraction of the latter.

Discussion

One of our major interests within the last 10 yr has involved structure-function investigations of hPRL *(11)*. As we previously reported, some features of PRL bioactivity vary depending on the origin of the receptor *(7)*. Since the most widely used bioassay for lactogenic hormones, including those of human origin *(1)*, involves rat Nb2 cells, the initial goal of our study was to set up homologous bioassays for human lactogens. We have described two new bioassays aimed at being used complementarily to, and certainly not to replace, the reference Nb2 assay. Obviously, establishing these new cell populations as alternative reference bio-

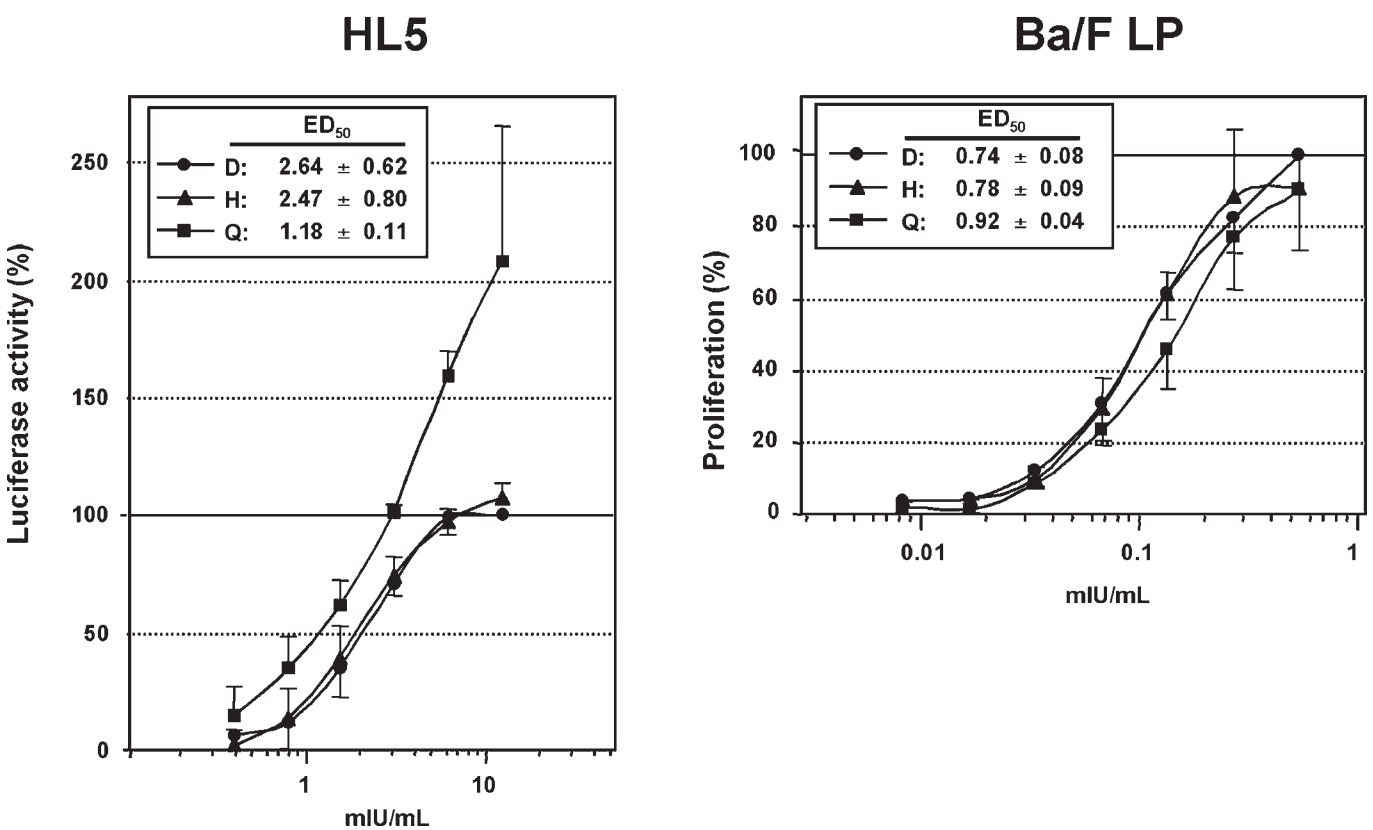


Fig. 5. Challenge of new homologous bioassays using unknown samples. Various coded hPRL samples (D, H, Q) were tested blind using HL5 and Ba/F3-LP cells. Results are expressed in percentage of maximal activity of sample D and represent values averaged from three independent experiments performed in duplicate (HL5) or triplicate (Ba/F3-LP). Samples D and H appeared to exhibit identical properties as reflected by their ED_{50} and amplitude of response. Sample Q was slightly less active (higher ED_{50}) in the proliferation assay but appeared to exhibit superagonistic properties in the transcriptional assay, both by its higher amplitude of response and by lower ED_{50}. See text for discussion.

assays would require more detailed investigations to assess intra- and interassay variability (especially along cell passages) and to better characterize specific properties of the ectopic hPRLR, such as receptor trafficking and processing, or signaling features compared to cells naturally expressing endogenous PRLR; such technical characterizations fall outside of the scope of the current study. While using these assays in routine structure-function studies, we were surprised by the very different results seen for a given analog, depending on whether it was characterized using HL5, T-47D, or Ba/F3-LP cells, all involving the hPRLR. Similarly, whereas G129R-hPRL achieves (sub-) maximal activity in the Nb2 assay *(12)*, its agonistic effect only reaches a plateau at ~6% of hPRL luciferase activity using 293 HEK transiently expressing the rat Nb2 receptor *(7)*. Moreover, the antagonistic properties of this analog are clearly observed in the luciferase assay while they fail to be detected in the Nb2 proliferation assay. A similar paradox has been reported by us and others for G120R and G120K-hGH analogs depending on the bioassay used *(13,14,17,24)*.

All these observations indicated that other parameters than only the receptor origin influenced the nature of biologic responses and, consequently, our estimate of the activity of a given hormone. In other words, for those analogs displaying altered properties compared to WT hPRL, it appears that more than a single in vitro bioassay is required to reliably reflect their intrinsic biologic potency. We therefore performed a direct comparison of several lactogen analogs in the available bioassays to elucidate the reasons of such apparent discrepancies (Fig. 4).

A major lesson learned in the course of our studies is that the intrinsic characteristics of the various bioassays used to screen hormone analogs can dramatically affect their attributed properties. We rapidly suspected that the biologic responses observed were correlated to the sensitivity of the various bioassays, and a tentative hypothesis is proposed in Fig. 6. It is assumed that the formation of receptor dimers—the active form of PRL and GH receptors—follows a bell-shaped curve as a function of ligand concentrations (Fig. 6, top) *(28)*. Such a bell-shaped curve occurs because

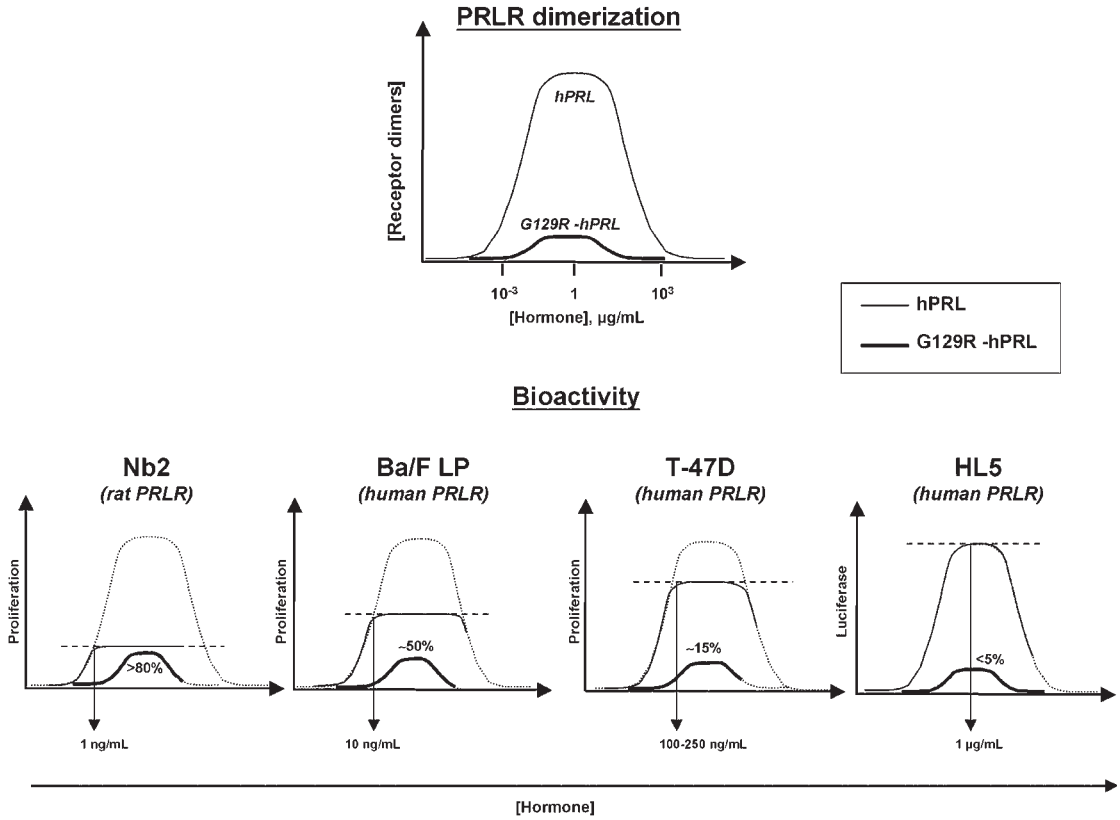


Fig. 6. Sensitivity of bioassays directly affects biologic properties of lactogens: hypotheses. (**Top**) The formation of receptor dimers displays a dose-dependent bell-shaped curve as previously demonstrated *(28)*. Because of alteration in binding site 2, G129R-hPRL exhibits a much lower ability to induce the formation of receptor dimers, so that the bell-shaped curve has a much lower amplitude *(38)*. (**Bottom**) The four bioassays used exhibit different sensitivities, as indicated by the maximal response achieved at hPRL concentrations gradually increasing from Nb2 to HL5 assays. Dotted lines schematically represent the dose-response formation of receptor dimers for hPRL and G129R analog (i.e., what is shown in the upper panel), whereas solid lines (thin for hPRL, thick for G129R-hPRL) schematically represent biologic responses. The horizontal hatched line represents the threshold of receptor dimers sufficient to produce maximal response. Since this threshold gradually increases from Nb2 to HL5 cells, the low amount of receptor dimers induced by G129R-hPRL will give rise to gradually lower activity (from >80% in the Nb2 assay to <5% in the HL5 assay). Based on these hypotheses, a given lactogen analog will display agonistic or antagonistic properties, depending on the sensitivity of the assay being used. See text for discussion.

the binding site 1 of ligands (PRL, GH) has a higher affinity for the receptor than the binding site 2, which results in favoring the formation of site 1–mediated hormone-receptor interactions (complexes 1:1) at ligand saturation (right part of the curve). As a consequence, these hormones tend to self-antagonize at high concentrations *(19)*. It has been reported that maximal proliferation of Nb2 cells is achieved at low receptor occupancy *(2)*, suggesting that the threshold of activated (dimerized) receptors required to produce maximal proliferation (discontinuous horizontal lines in Fig. 6) is far below the situation in which entire receptor population is dimerized. Accordingly, maximal proliferation of Nb2 cells is observed at low concentrations of lactogen (1 ng/mL of hPRL) *(1)*, and although increasing ligand concentrations probably increases the number of receptor dimers at the cell surface (as represented by the dotted bell-shaped curve in Fig. 6, bottom left), it fails to further stimulate cell proliferation, which remains at the plateau. This threshold of ligand concentration at which "maximal bioactivity" occurs is higher for Ba/F3-LP cells (maximum at 10 ng/mL, this study), and still higher for T-47D cells (100–250 ng/mL) *(25)*.

The molecular explanations underlying the different sensitivities of these bioassays are still unclear. They are presumably independent of receptor affinity, since both rat (Nb2) and human (other assays) PRLRs exhibit a similar affinity for hPRL. They also do not seem to be correlated with the level of receptor expression, since Nb2 and T-47D cells express >10,000 PRLRs per cells *(2,25)*, whereas Ba/F3-LP cells express <300 receptors. It is possible that the assay sensitivity involves the paradigm of "minimal time of homodimer persistence" recently hypothesized by Gertler and Djiane *(29)*, which remains to be investigated using our bioassays.

Finally, it is reasonable to postulate that intrinsic constraints of intracellular limiting factors prevent cells to react above a certain level of receptor stimulation, i.e., that there

is a threshold of stimulation above which cells cannot divide more quickly. In contrast to proliferation assays, the luciferase assay is probably not affected by limiting factors to such an extent, since the biologic response involves a minimal number of intracellular molecules (Fig. 1) and induces the expression of a single protein (luciferase). Therefore, in this assay, the bioactivity curve more closely parallels that of receptor dimerization (Fig. 6). This correlates well with the fact that maximal activity is achieved at hPRL concentrations 3 log units higher than in the Nb2 assay (Fig. 6, right).

In summary, our hypothesis suggests that the dose-response curves of bioactivity follow that of receptor dimer formation until the threshold of dimers leading to maximal response is achieved; beyond this concentration, the bioactivity curve remains at a plateau (Fig. 6, bottom). Using HL5 cells, bell-shaped curves are observed in bioassays, probably reflecting at the activity level the phenomenon of ligand self-antagonism on the formation of receptor dimers (see above). For the other assays, bell-shaped curves are observed when it is possible to assay hormone concentrations high enough to decrease the number of receptor dimers below the threshold necessary to produce a maximal biologic response (Fig. 6, bottom). Since this threshold concentration is higher for Ba/F3-LP cells than for Nb2 cells, bell-shaped curves are more easily observed with the former than the latter. The more sensitive the assay, the more difficult it is to obtain bell-shaped curves.

This hypothesis also helps one to understand the versatile bioactivity of G129R-hPRL depending on the assay used. Binding site 2 of this analog is clearly altered, but site 2 binding is not completely abolished *(12)*. Hence, G129R-hPRL is still able to induce the formation of low levels of receptor dimers (Fig. 6, top), which is sufficient to induce (sub-)maximal proliferation of Nb2 cells, whose proliferation requires only low levels of receptor activation (Fig. 6, bottom left). By contrast, in the luciferase assay, which appears to more directly reflect the amount of PRLR dimerized, the activity of G129R-hPRL was almost nil (2 to 3% of hPRL) (Fig. 6, bottom right). In the two other assays whose sensitivity is intermediate (Ba/F3-LP and T-47D), G129R-hPRL exhibited partial agonistic activity, which is inversely proportional to the threshold of maximal hPRL activity. In agreement with these observations, the antagonistic activity of this analog will directly depend on its intrinsic agonistic activity in each bioassay. In this respect, it is noteworthy that a slope down in competition assays can thus be misinterpreted to confer antagonistic properties to a lactogen analog. This is the case for S179D-hPRL in the Ba/F-LP or Nb2 assays, where it shows intrinsic agonistic activity and displays self-antagonism at extremely high concentrations (Fig. 4B; *[23]*). The same observation also applies to G129R-hPRL in sensitive bioassays.

Transient transfection of cells using plasmids encoding receptor and reporter genes is a methodology extensively used to investigate structure-function relationships of ligands or receptors. One advantage of generating stable "HL" clones is that the assay can be miniaturized into 96-well plates, which is less time-consuming, requires fewer cells and hormones, and does not require normalization of RLU values by another enzymatic activity, such as β-galactosidase. Such combined technical improvements provide more reproducible results, as demonstrated in our study when coded samples provided by the WHO were tested. Perhaps more important, our luciferase assay also provides some information not provided by other classic bioassays. For example, although S179D-hPRL would be considered a weak agonist based on the shift (to the right) of its dose-response curves in proliferation assays (Fig. 4), it appears from using clone HL5 that this analog is actually able to stimulate the PRLR more efficiently than hPRL itself, since the amplitude of its response is higher, which was unexpected *(23)*. The same observation is true for glycosylated hPRL (sample Q, Fig. 5), which was reported to be a less active mitogen than nonglycosylated hPRL *(22)*. Although the molecular bases underlying these observations are still to be explored, this clearly suggests that using HL clones can open new doors of investigation in structure-function studies of lactogens. In this respect, it will be of interest to further characterize other HL clones, such as HL10, which appears to be more sensitive to low concentrations of ligands.

In conclusion, although it is common in the field of experimental research to consider biologic assays as routine tools to be used as "kits" in molecular biology—that is, by following the protocols established, we believe that the various lactogen bioassays should in fact be used very carefully, keeping in mind their intrinsic characteristics. Therefore, the results obtained from in vitro assays should be cautiously interpreted, since our data demonstrate to what extent the intrinsic features of bioassays can affect the nature and the amplitude of the biologic responses observed. The Nb2 cell proliferation assay is extremely sensitive and should be used to assess the agonistic properties of PRL/GH/PL analogs, especially to identify those suspected to exhibit only residual activity *(24)*. The assay involving Ba/F3-LP cells is similar to the Nb2 assay, with the one advantage that the response is mediated by the homologous receptor when using human lactogens. By contrast, the luciferase assay is obviously less sensitive and is therefore less appropriate for detecting low levels of agonistic activity. However, since it is less dependent on limiting factors, it allows the identification of any change in the mechanism of receptor activation. This includes lactogen analogs displaying antagonistic properties, such as G129R-hPRL and G120K-hGH, or in contrast analogs exhibiting superagonistic properties (i.e., leading to maximal activity, higher in amplitude to that of natural lactogens), such as S179D-hPRL or glycosylated hPRL.

Beyond understanding the molecular mechanisms of receptor activation, in vitro bioassays are also used to identify the analogs within a set of hormone mutants that should

be tested in vivo. Thus, the present study raises the question of which bioassay is the most accurate to predict the in vivo biologic properties of a lactogen analog. For example, whereas contradictory observations have been published regarding the agonistic and antagonistic activities of G120R(or K)-hGH in vitro *(13,14)*, this analog fails to completely abolish PRLR-mediated actions in vivo, since transgenic mice overexpressing G120R-hGH are fertile and lactate *(30)*, in contrast to what would be expected if inhibition of PRLR actions was as efficient as in PRLR knockout mice *(31)*. This suggests that the residual in vitro agonistic activity reported by Dattani et al. *(13)* is the relevant activity in the in vivo context. The same discrepancy between in vitro and in vivo data also applies to G129R-hPRL and S179D-hPRL, both of which appear to exhibit agonistic properties in some, if not all, in vitro bioassays used in our study, but were nevertheless reported to exhibit antagonistic properties in vivo *(32,33)*. This highlights that no single bioassay is perfect and can exhaustively cover all aspects of the biologic properties of lactogen analogs. As an example of these limitations, our transfection-based assays reflect only part of the picture since a single form of the receptor, the long PRLR, is expressed. Obviously, the in vivo context is much more complex since many isoforms of the PRLR, differing by their signaling properties, have now been identified in several species, including humans *(34,35)*.

Our observations emphasize the necessity to use more than a single bioassay and, still more important, to perform both agonism and antagonism assays in parallel to accurately evaluate antagonistic properties of a given analog. Our deep feeling is that a potent antagonist is an analog devoid of residual agonistic activity in any lactogenic bioassay, especially in the most sensitive ones, proliferation of Ba/F3-LP and of Nb2 cells.

Materials and Methods

Hormones

Recombinant hPRL, G129R-hPRL, and S179D-hPRL were prepared as previously described *(36)*. Biosynthetic hGH (lot 7AFE1653) was obtained from Lilly France S.A. (Saint-Cloud, France), and extractive hPL and recombinant bGH were kindly provided by Dr. A. F. Parlow (National Hormone and Pituitary Program, Torrance, CA). Recombinant oPL and oPRL were generous gifts of Dr. A. Gertler (Hebrew University of Jerusalem, Rehovot, Israel) and G120K-hGH was kindly provided by Dr. W. Bennett (Sensus). Coded hPRL hormone preparations (named D, H, and Q) were provided by Dr. B. Rafferty (National Institute for Biological Standards and Control, Herts, UK) on behalf of the WHO. These hormone preparations were obtained from conditioned media of murine C127 cells transfected with the hPRL coding sequence, and purified as described previously *(22)*.

Transcriptional Assay (Clone HL5)

Transfection and Clonal Selection

We used the HEK fibroblast 293 cell line to generate clonal cell lines stably expressing the hPRLR, since this cell line has been shown to highly express cDNAs controlled by the cytomegalovirus (CMV) promoter *(7,37)*. Cells were routinely cultured in DMEM-Nut F12 medium supplemented with 10% FCS, 2 m*M* glutamine, 50 U/mL of penicillin, 50 μg/mL of streptomycin (Invitrogen Life Technologies, France). Cells (5×10^6 in 10-cm Petri dishes) were transfected using two pCDNA3 plasmids encoding the hPRLR and the LHRE-luciferase reporter genes (2 or 0.4 μg each) using the calcium-phosphate precipitate procedure *(7,37)*. Twenty-four hours after transfection, cells were shifted to growth medium containing 500 μg/mL of active G-418 (geneticin) for clone selection. From this step, G-418 was systematically added to all culture media. After 15–20 d, single G-418-resistant colonies were localized by microscope, picked out individually by local trypsinization, and amplified in 24-well plates before being characterized for their ability to respond to hPRL as monitored by the induction of luciferase activity. Thirty-two clones were isolated (eight of which were obtained from plates transfected using 0.4 μg of each plasmid) and referred to as HL1 to HL32.

Luciferase Assay

After trypsinization, cells were counted and aliquoted in 96-well plates at a density of 50,000 cells/(100μL/well). Plating medium contained 0.5% FCS to allow cell adhesion. Six to 18 h (overnight) after plating, 100 μL of two-fold concentrated hormones diluted in FCS-free medium was added to each well. Typically, we assayed the different hormones (WT or analogs) at concentrations ranging from ~10 ng/mL to ~250 μg/mL (~10 μ*M*) in duplicate. After 18–24 h of stimulation, culture medium was aspirated and cells were lysed for at least 10 min in 50 μL of lysis buffer (Promega, Charbonnieres-Les-Bains, France). Luciferase activity for each experimental condition was counted in 10–20 μL of cell lysates for 10 s using a luminometer (Lumat LB 9501; Berthold, Nashua, NH). The difference between duplicates never exceeded 15% of RLU values. Although the latter were found to slightly decrease along cell passages (which presumably greater reflects senescence of 293 cell cultures classically observed after 15–20 passages than a strictly PRLR-dependent effect), this did not significantly affect the fold induction of luciferase activity (calculated as the ratio between the RLU of stimulated vs nonstimulated cells), which always remained around 10 or higher.

Proliferation Assay (Ba/F3-LP and HP)

Transfection, Selection, and Proliferation Assay

Ba/F3 is a pro-B murine cell line dependent on IL-3 for growth. Cells were transfected by electroporation using 20 μg of plasmid encoding the hPRLR (CMV promoter),

and then the population stably expressing the receptor was selected by several passages in geneticin-containing medium. Ba/F3-hPRLR cells were routinely maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FCS; 2 m*M* glutamine; 50 U/mL of penicillin; 50 µg/mL of streptomycin; 700 µg/mL of G-418; and 10 (population LP), 100 (population MP), or 1000 ng/mL (population HP) of WT hPRL instead of IL-3. Before proliferation assays, cells were starved for 6 h in 1% FCS RPMI-1640 medium with additives, then distributed in 96 well-plates at a density of 5×10^4 cells/well in a final volume of 100 µL. One hundred microliters of (2X) hormones diluted in the same medium were added after the starvation period. Cell proliferation was estimated after 3 d of hormonal stimulation by adding 10 µL of WST-1 tetrazolium salt. OD at 450 nm (OD_{450}) was measured after 1–3 h of colorimetric reaction using an enzyme-linked immunosorbent assay plate reader. The experiments were routinely performed at least three times in triplicate or quadruplicate (variability usually does not exceed 10% provided peripheral wells are avoided to circumvent edge effects). Experiments of cell dilutions showed that the ratio of cell density/OD is almost linear in the range of 50,000–150,000 cells/well, which corresponds to cell densities achieved in this bioassay.

Other Cell Studies

The Nb2 cell line was routinely maintained in RPMI-1640 supplemented with 10% horse serum (HS), 10% heat-inactivated FCS, 2 m*M* glutamine, 50 U/mL of penicillin, 50 µg/mL of streptomycin, and 100 µ*M* β-mercaptoethanol. T-47D cells were routinely cultured in DMEM/F12 (1:1) medium containing 10% FCS, 2 m*M* glutamine, 50 U/mL of penicillin, and 50 µg/mL of streptomycin. Proliferation assays using Nb2 and T-47D cells were performed as previously described *(23)*, and binding experiments and FACS analyses were performed following protocols detailed elsewhere *(19,23,25)*.

Acknowledgments

We are grateful to F. Ferrag for help with the Ba/F3 cell transfections. We acknowledge Drs. A. F. Parlow, A. Gertler, and W. Bennett for providing some of the hormones. We are also grateful to Dr. B. Rafferty for permission to use some of our data obtained in the collaborative WHO study.

References

1. Tanaka, T., Shiu, R. P., Gout, P. W., Beer, C. T., Noble, R. L., and Friesen, H. G. (1980). *J. Clin. Endocrinol. Metab.* **51,** 1058–1063.
2. Shiu, R. P., Elsholtz, H. P., Tanaka, T., et al. (1983). *Endocrinology* **113,** 159–165.
3. Goffin, V., Norman, M., and Martial, J. A. (1992). *Mol. Endocrinol.* **6,** 1381–1392.
4. Luck, D. N., Gout, P. W., Beer, C. T., and Smith, M. (1989). *Mol. Endocrinol.* **3,** 822–831.
5. Gertler, A., Shamay, A., Cohen, N., et al. (1986). *Endocrinology* **118,** 720–726.
6. Clevenger, C. V., Freier, D. O., and Kline, J. B. (1998). *J. Endocrinol.* **157,** 187–197.
7. Goffin, V., Kinet, S., Ferrag, F., Binart, N., Martial, J. A., and Kelly, P. A. (1996). *J. Biol. Chem.* **271,** 16573–16579.
8. Ali, S., Pellegrini, I., Edery, M., et al. (1991). *J. Biol. Chem.* **266,** 20110–20117.
9. Shirota, M., Banville, D., Ali, S., et al. (1990). *Mol. Endocrinol.* **4,** 1136–1143.
10. Ali, S., Edery, M., Pellegrini, I., et al. (1992). *Mol. Endocrinol.* **6,** 1242–1248.
11. Kinet, S., Bernichtein, S., Llovera, M., Kelly, P. A., Martial, J. A., and Goffin, V. (2001). *Recent Res. Dev. Endocrinol.* **2,** 1–24.
12. Goffin, V., Struman, I., Mainfroid, V., Kinet, S., and Martial, J. A. (1994). *J. Biol. Chem.* **269,** 32,598–32,606.
13. Dattani, M. T., Hindmarsh, P. C., Brook, C. D., Robinson, I. F., Kopchick, J. J., and Marshall, N. J. (1995). *J. Biol. Chem.* **270,** 9222–9226.
14. Fuh, G., Colosi, P., Wood, W. I., and Wells, J. A. (1993). *J. Biol. Chem.* **268,** 5376–5381.
15. Boutin, J. M., Edery, M., Shirota, M., et al. (1989). *Mol. Endocrinol.* **3,** 1455–1461.
16. Das, R. and Vonderhaar, B. K. (1997). *J. Mammary Gland Biol. Neopl.* **2,** 29–39.
17. Fuh, G. and Wells, J. A. (1995). *J. Biol. Chem.* **270,** 13133–13137.
18. Graham, M. L., Krett, N. L., Miller, L. A., et al. (1990). *Cancer Res.* **50,** 6208–6217.
19. Kinet, S., Bernichtein, S., Kelly, P. A., Martial, J. A., and Goffin, V. (1999). *J. Biol. Chem.* **274,** 26033–26043.
20. Finidori, J. and Kelly, P. A. (1995). *J. Endocrinol.* **147,** 11–23.
21. Jeay, S., Sonenshein, G., Postel-Vinay, M. C., and Baixeras, E. (2000). *Mol. Endocrinol.* **14,** 650–661.
22. Price, A. E., Logvinenko, K. B., Higgins, E. A., Cole, E. S., and Richards, S. M. (1995). *Endocrinology* **136,** 4827–4833.
23. Bernichtein, S., Kinet, S., Jeay, S., et al. (2001). *Endocrinology* **142,** 3950–3963.
24. Goffin, V., Bernichtein, S., Carrière, O., Bennett, W. F., Kopchick, J. J., and Kelly, P. A. (1999). *Endocrinology* **140,** 3853–3856.
25. Llovera, M., Pichard, C., Bernichtein, S., et al. (2000). *Oncogene* **19,** 4695–4705.
26. Chen, T. J., Kuo, C. B., Tsai, K. F., Liu, J. W., Chen, D. Y., and Walker, A. M. (1998). *Endocrinology* **139,** 609–616.
27. Fuh, G., Cunningham, B. C., Fukunaga, R., Nagata, S., Goeddel, D. V., and Wells, J. A. (1992). *Science* **256,** 1677–1680.
28. Ilondo, M. M., Damholt, A. B., Cunningham, B. A., Wells, J. A., De Meyts, P., and Shymko, R. M. (1994). *Endocrinology* **134,** 2397–2403.
29. Gertler, A. and Djiane, J. (2002). *Mol. Genet. Metab.* **75,** 189–201.
30. Chen, W. Y., Chen, N. Y., Yun, J., Wagner, T. E., and Kopchick, J. J. (1994). *J. Biol. Chem.* **269,** 15892–15897.
31. Ormandy, C. J., Camus, A., Barra, J., et al. (1997). *Genes Dev.* **11,** 167–178.
32. Chen, N. Y., Holle, L., Li, W., Peirce, S. K., Beck, M. T., and Chen, W. Y. (2002). *Int. J. Oncol.* **20,** 813–818.
33. Kuo, C. B., Wu, W., Xu, X., et al. (2002). *Cell Tissue Res.* **309,** 429–437.
34. Kline, J. B., Rycyzyn, M. A., and Clevenger, C. V. (2002). *Mol. Endocrinol.* **16,** 2310–2322.
35. Hu, Z. Z., Meng, J., and Dufau, M. L. (2001). *J. Biol. Chem.* **276(44),** 41086–41094.
36. Paris, N., Rentier-Delrue, F., Defontaine, A., et al. (1990). *Biotechnol. Appl. Biochem.* **12,** 436–449.
37. Lebrun, J. J., Ali, S., Goffin, V., Ullrich, A., and Kelly, P. A. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 4031–4035.
38. Goffin, V., Shiverick, K. T., Kelly, P. A., and Martial, J. A. (1996). *Endocr. Rev.* **17,** 385–410.