A STRUCTURE - FUNCTION ANALYSIS OF BOVINE PROLACTIN

by

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ABSTRACT

The 23 kDa pituitary protein hormone bovine prolactin (bPRL) belongs to a family of hormones including growth hormones, prolactins, and placental lactogens, and is involved in regulating a variety of physiological processes including lactation and immune response. The biological actions of bPRL are mediated by cell surface receptors and it is anticipated, as reported for human growth hormone (hGH) and based on sequence homology among members of this hormone family, that the hormone-receptor interaction involves receptor dimerization by a single hormone molecule. The structure of bPRL is not known, but is believed to resemble that of hGH in consisting of four α-helices arranged in a left twisted helical bundle. Variants of recombinant methionyl bovine prolactin (met-bPRL) containing single amino acid changes were generated by site-directed mutagenesis in order to carry out a structure-function analysis of the interaction of bPRL with the Nb2 PRL receptor.

The changes involved the replacement, in most cases with alanine, of residues in the loop region joining putative helices 1 and 2, in putative helix 3, and in putative helix 4; regions homologous with functionally important portions of closely related proteins including human prolactin (hPRL) and hGH. The variant proteins were produced as inclusion bodies in E. coli, extracted with N-lauryl sarcosine (sarcosyl), and renatured by air oxidation at pH 10. The contribution of the residues to the biological activity of bPRL was assessed using the Nb2 lymphoma cell bioassay. In this assay, in which met-bPRL was as active as pituitary-derived bPRL, growth factor activity is measured, and the rate of cell proliferation is proportional to the concentration of lactogen present. The findings are discussed in the light of a putative three-dimensional structure of bPRL, modelled using the structural coordinates of hGH.

None of the exchanges of residues of the loop region resulted in drastic reductions in the mitogenic activity. The variants H59A, L63A, Q71V, Q73V, and Q74V exhibited an approximate two- to three-fold reduction in bioactivity compared to unmodified met-bPRL, suggesting that these residues may be involved in the mitogenic activity of bPRL. Of the 18 putative helix 4 residues examined only substitutions of R177 and K181 led to marked decreases
in mitogenic activity, indicating that these residues are very important to the bioactivity of bPRL. Replacements of L171, R176, and D183 resulted in variants with bioactivities 2.5- to 3-fold less than that of unmodified met-bPRL and only 9% activity was exhibited by the mutant D178E. However, according to the putative model of bPRL the side chains of H59, L63, L171, and D178 point into the molecule; thus, these residues may play a structural role in maintaining the shape of the binding site rather than directly contacting the receptor. The variant G129R had no mitogenic activity, and E128A had reduced bioactivity, suggesting that bPRL contains a second receptor binding site analogous to that described for hGH.

Comparison of these results with those reported for related hormones has confirmed that the bPRL residues involved in mediating the mitogenic activity of the hormone, and presumably also in interacting with the Nb2 PRL receptor, are located in regions similar to those identified for other hormones. However, many differences exist in that the specific residues involved in eliciting the biological actions are not at equivalent positions; these differences likely contribute to the specificity of hormone-receptor interactions. The results of this study thus not only provide insight into the interaction between bPRL and the Nb2 PRL receptor, but also, when considered in light of the putative three-dimensional structure of bPRL and with respect to related hormones, help to give a greater understanding of the general mechanism of receptor binding by members of the growth hormone/prolactin/placental lactogen family of hormones.
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LIST OF ABBREVIATIONS

A$_{260}$  absorbance at 260 nm
amp        ampicillin
bGH        bovine growth hormone
bp         base pair
bPRL       bovine prolactin
dH$_2$O     distilled water
DTT        dithiothreitol
EDTA       ethylenediaminetetraacetic acid
hGH        human growth hormone
hGHbp      human growth hormone binding protein
hPL        human placental lactogen
hPRL       human prolactin
hPRLbp     human prolactin binding protein
IPTG       isopropyl-β-D-thiogalactopyranoside
kDa        kilodalton
mPL-II     mouse placental lactogen II
OD$_{600}$  optical density at 600 nm
oPL        ovine placental lactogen
oPRL       ovine prolactin
pGH        porcine growth hormone
PCR        polymerase chain reaction
PRL-R      prolactin receptor
sarcosyl   N-lauryl sarcosine
SDS-PAGE   sodium dodecyl sulfate polyacrylamide gel electrophoresis
U          unit
vol        volume
DEDICATION

To my mother, Sandra Anne Huyer (1935 - 1992)

With all my love.
Molecular recognition is a key component of many biochemical processes, including DNA-protein interactions, ligand-receptor binding, and enzyme-substrate recognition. All of these systems require very selective recognition mechanisms to maintain the proper interactions. This selectivity is often exquisite, permitting the discrimination of molecules or targets that are structurally very similar. The bases of this specificity are often poorly understood, and a great deal of scientific effort has been and continues to be aimed towards understanding the problem.

A classic example of molecular recognition is ligand-receptor binding, such as peptide hormones binding to their receptors. With the relative ease of molecular biology techniques, it has been possible to carry out structural and functional studies to delineate the contact surfaces between the proteins, to reveal the individual amino acids involved in the interactions, and to determine the relative importance of each interaction. This type of approach has been applied here to carry out a structure-function analysis of bovine prolactin as it interacts with the prolactin receptors on Nb2 lymphoma cells which depend on prolactin for growth.

1.1 Biological Actions of Prolactin

The pituitary gland is the source of a number of important hormones including growth hormone and prolactin. Prolactin, which is now known to exist in all classes of vertebrates, was first identified as a pituitary factor with the capability to induce milk secretion in rabbits (Stricker and Grueter, 1928). Soon afterwards it was discovered that proliferation of the mammary gland and lactation could be induced in spayed virgin rabbits by injecting the rabbits with extracts of sheep pituitaries (Corner, 1930). In addition, extracts of bovine pituitaries were able to induce the growth of pigeon crop sacs (Riddle and Braucher, 1931). A search for the pituitary factor causing these effects led to the identification of a previously unknown hormone produced by the anterior pituitary; this hormone was named prolactin (Riddle et al., 1932).

Prolactin, like growth hormone, is synthesized in acidophilic cells of the pituitary (prolactin in lactotroph cells, growth hormone in somatotroph cells) as a precursor, pre-prolactin,
with a signal peptide of approximately 25 amino acids attached to the N-terminus. As found for
pre-growth hormones, the signal peptide directs pre-prolactin to the endoplasmic reticulum of the
acidophilic cells where rapid processing to the mature hormone occurs (Wallis et al., 1985).
Secretion of the anterior pituitary hormones is controlled by factors from the hypothalamus, such
as dopamine; however, in contrast to growth hormone, control of prolactin secretion in mammals
is largely inhibitory. That is, removal of the hypothalamus results in marked increases in
prolactin secretion whereas the release of other pituitary hormones, including growth hormone,
decreases. In birds and reptiles, however, prolactin is under positive regulatory control of the
hypothalamus. Many different factors including other hormones and neural signals are involved
in the physiological control of the secretion of both growth hormone and prolactin. Thus,
regulation of secretion of these hormones is very complex and, indeed, is not yet fully
understood (Wallis et al., 1985).

Prolactin is a member of a large family of hormones that also includes growth hormone and
placental lactogen. Sequence analysis has confirmed that these hormones are evolutionarily
related, and it is thought that a single ancestral gene diverged to give rise to the separate prolactin
and growth hormone lineages while genes for the placental lactogens may have arisen from both
these lineages (Miller and Eberhardt, 1983; Nicoll et al., 1986). These globular polypeptide
hormones are found in species ranging from teleost fish to primates and as a group regulate a
large number of biological processes. Growth hormones have been shown to have somatogenic,
anabolic, lipolytic, insulin-like, and diabetogenic effects (Chawla et al., 1983), while placental
lactogens, although their role has been less well defined, seem to be involved in regulating
lactation, fetal growth, and fetal metabolism (Brinck-Johnsen and Benirschke, 1982; Ogren and
Talamantes, 1988). More than 85 biological functions have been described for prolactin (Nicoll
and Bern, 1972) including such diverse processes as osmoregulation, amphibian metamorphosis,
development of the incubation patch of birds and seahorses, and hyperplasia of the crop sac
mucosa in pigeons.

In mammals, some of the most important physiological effects of prolactin relate to
lactation, to growth and development of the mammary gland, and to immune stimulatory
activities (Clarke and Bern, 1980). Prolactin acts in concert with insulin and glucocorticoids to stimulate milk protein gene expression and has been shown to influence the rate of gene transcription and the stability of the milk protein mRNAs (Guyette et al., 1979). Growth hormones from primates also have lactogenic activity (Li, 1973), indicating some overlap between the functions of growth hormone and prolactin in these species. In fact, the lactogenic activity of growth hormone from humans is such that, until human prolactin was purified in 1972 (Hwang et al., 1972), many researchers doubted that humans produced both of these hormones. Prolactin and growth hormone are also involved in the regulation of the immune system (Gala, 1991) and cells involved with immunity, such as lymphocytes, express the genes for these hormones and for their receptors. Other evidence suggests that growth hormone and prolactin have roles as hematopoietic growth and differentiation factors and thus they can be considered as having autocrine or paracrine roles in addition to their well-established endocrine actions (Gala, 1991; Hooghe et al., 1993).

As described above, prolactin exerts a strong effect on a number of physiological processes and has the ability to promote cell growth and/or differentiation in various tissues. The mitogenic effects of prolactin have been utilized in the development of a sensitive bioassay for prolactin and other lactogenic hormones. Proliferation of cultured cells derived from a transplantable lymphoma, designated Nb2 lymphoma, that arose in the lymph nodes of an estrogenized male rat (Nb strain) requires the presence of lactogens, and the rate of cell proliferation is proportional to the concentration of lactogen in the range of 5 - 500 pg/mL (Gout et al., 1980). Thus, the Nb2 cell line provides a sensitive and highly specific in vitro bioassay for lactogenic hormones, including prolactin (Tanaka et al., 1980). Prior to the development of the Nb2 cell assay, bioassays commonly used to determine levels of prolactin included the pigeon crop sac assay (Nicoll, 1967) and in vitro assays employing mammary gland cultures (Forsyth and Myres, 1971; Kleinberg and Frantz, 1971; Loewenstein et al., 1971; Turkington, 1971). Since these assays are more time-consuming to perform and do not exhibit the same sensitivity to levels of prolactin as the Nb2 cell assay, the latter assay has become the method of choice for determining concentrations and specific activity of biologically active lactogens.
1.2 Hormone Structure

In addition to the many biological features which prolactin, growth hormone, and placental lactogen have in common, there are many shared sequences at the amino acid level (Nicoll et al., 1986; Wallis, 1978). These similarities have led to the grouping of these proteins into a single hormone family. In turn, this family of hormones and the receptors with which they interact are part of a larger cytokine-hormone superfamily, based on homology of the receptors (Bazan, 1989, 1990a,b; Kelly et al., 1991). This family includes the interleukins, granulocyte-macrophage colony-stimulating factors (GM-CSF and G-CSF) and erythropoietin (EPO). In spite of the fact that the degree of sequence similarity between the proteins in the larger superfamily is small, the sequence features which are shared suggest that their structures have a related three-dimensional fold of four-helix bundles.

Although there is a great deal of interest in determining the structural features of the prolactins, growth hormones, and placental lactogens which give rise to their various biological effects, to date only the three-dimensional structures of porcine growth hormone (pGH) (Abdel-Meguid et al., 1987) and human growth hormone (hGH) (de Vos et al., 1992; Ultsch et al., 1994) have been solved by X-ray crystallography. Both proteins are 191 amino acids in length and have molecular weights of 22 kDa. The structure of pGH was solved to 2.8 Å resolution and revealed a molecule containing four α-helices arranged in a left twisted helical bundle. Although the tightly packed helix bundle has a similar structure to that of other proteins such as cytochrome b-562 (Lederer et al., 1981), cytochrome c' (Weber et al., 1980), and myohemerythrin (Hendrickson and Ward, 1977), the connectivity of the helices is unusual. Helices 1 and 2 of pGH are parallel to each other and antiparallel to helices 3 and 4. In order to achieve this arrangement, long loop regions link the two sets of parallel helices while a short segment connects helix 2 to helix 3 (Figure 1). Porcine growth hormone was the first molecule for which this type of connectivity was demonstrated, and because of the sequence homology between the growth hormones, placental lactogens, and prolactins, it is believed that this is the general three-dimensional structure for this family of hormones. Recently, the elucidation of structures for other members of the larger cytokine superfamily including IL-2, hGH, IL-4,
GM-CSF, has confirmed the generality of this type of fold (Bazan and McKay, 1992; de Vos et al., 1992; Diederichs et al., 1991; Powers et al., 1992; Smith et al., 1992; Walter et al., 1992). Other structural features of pGH include a bend in helix 2 at P89, and disulfide loops which connect C53 in the first crossover connection to C164 in helix 4 and C181 in helix 4 to C189.

Figure 1. Representation of the structure of porcine growth hormone (pGH). The four α-helices are represented by cylindrical rods and non-helical regions are shown as thin tubes. Helix 1 is blue, helix 2 is pink, helix 3 is green, and helix 4 is orange. The N-terminus is located in the upper left-hand corner and the C-terminus in the lower left-hand corner. The disulfide shown links the centre of the loop joining helices 1 and 2 with the centre of helix 4. A hidden disulfide joins two cysteine residues in the region between helix 4 and the C-terminal. The arrows point toward the position where introns have been found within the coding sequence. The figure is taken from Abdel-Meguid et al., 1987.
The structure of hGH bound to the extracellular domain of its receptor (de Vos et al., 1992) is almost identical to that of free pGH. Differences exist in the structures of the connecting loops: hGH has two small helical segments (K38-N47 and R64-K70) in the loop joining helices 1 and 2 and a small helix from R94-S100 in the loop between helices 2 and 3. The first two helical segments were not observed for pGH. These two minihelices of hGH are involved in contacts with the receptor and it was originally speculated that they may represent conformational changes in the hormone due to receptor binding (de Vos et al., 1992). However, crystal structures of two hGH variants not complexed to receptors also reveal the existence of these minihelices and suggest that these are characteristic features of hGH (Ultsch et al., 1994). In contrast to the small helical section in the loop joining helix 2 to helix 3 in hGH, an omega-loop conformation was described for this section of pGH (Abdel-Meguid et al., 1987). The reason for this apparent structural difference is not clear, since the sequences of pGH and hGH are almost identical in this region and this segment is apparently not involved in receptor binding. However, since the structure of hGH was refined to an R factor of 0.204 while the R factor for the pGH structure was 0.33, the difference in the conformation of the loop joining helices 2 and 3 may simply reflect the more preliminary state of refinement of the pGH molecule (Wells and de Vos, 1993) (R factor is the residual disagreement between the experimentally observed diffraction amplitudes and those calculated for a model crystal: values range from 0.0 for exact agreement to around 0.59 for total disagreement).

Refined structures of other members of the cytokine superfamily have recently been reported and these show significant resemblances to each other (Wlodawer et al., 1993). Superimpositions of the available structures indicate that the greatest degree of structural homology is in the four-helix bundle, with helix 4 being the most highly conserved structural element. Each cytokine has a hydrophobic core within the interior of the four-helix bundle and although there is very little sequence conservation among the residues forming the core, there is a great deal of structural similarity. In addition, there is significant sequence and structural similarity between the extracellular domains of cytokine receptors which are involved in binding hematopoietins (Bazan, 1990b). Based on the similarities between the receptors as well as
between the ligands, it has been suggested that the overall mode of binding between the ligands and the receptors might be very similar within the cytokine superfamily (Wlodawer et al., 1993).

1.3 Receptors

The initial step in the action of growth hormones, prolactins, and placental lactogens is the binding of the hormone to cell surface receptors. Once bound, intracellular signals are generated which initiate the cellular response. As noted above, these proteins are members of a single hormone family which is itself part of a large cytokine hormone family. The superfamily was recognized on the basis of receptor homologies, not on the basis of hormone and cytokine sequences; therefore, it is not surprising that the receptors for this group of related proteins form a family. In addition to the receptors for growth hormone and prolactin, this family includes the receptors for granulocyte colony-stimulating factor, erythropoietin, granulocyte-macrophage colony-stimulating factor, the interleukins IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, the β- and γ-chains of the IL-2 receptor, as well as a number of other proteins (Bazan, 1989, 1990b; D'Andrea et al., 1989; de Vos et al., 1992; Fukunaga et al., 1990; Gearing et al., 1989; Goodwin et al., 1990; Idzerda et al., 1990; Itoh et al., 1990; Kelly et al., 1991; Pathy, 1990; Savino et al., 1993). The receptors in this family are single subunit proteins composed of an extracellular domain which interacts with the activating ligand, a short transmembrane section, and a cytoplasmic domain (Figure 2). Most of the diversity between these related receptors is seen in the cytoplasmic portion of the proteins. Since all of the members of this receptor family are found on, and mediate effects on, cells of the hematopoietic lineage, it has been suggested that this group of proteins be referred to as the hematopoietin receptor superfamily (Idzerda et al., 1990).
Figure 2. Schematic representation of members of the cytokine/hematopoietin receptor superfamily. Abbreviations: GHbp, growth hormone binding protein; GH-R, growth hormone receptor; PRL-R, prolactin receptor; EPOR, erythropoietin receptor; IL-2R, interleukin-2 receptor; IL-3R, interleukin-3 receptor; IL-4R, interleukin-4 receptor; IL-5R, interleukin-5 receptor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; IL-6R, interleukin-6 receptor; IL-7R, interleukin-7 receptor; IL-9R, interleukin-9 receptor; G-CSFR, granulocyte colony-stimulating factor receptor. The Nb2 form of the PRL receptor is due to a mutation in the PRL receptor gene which results in the loss of 594 bp from the cytoplasmic domain of the long form of the PRL-R. The thin black lines indicate the conserved cysteines; the thick black lines indicate the WSXWS motif; the transmembrane region is stippled; and the proline-rich motif (PRM) is indicated by a hatched box. Figure is adapted from Kelly et al., 1994.
1.3.1 Extracellular Domains

Although the sequence homology between the extracellular, hormone/growth factor binding region of these receptors is low (14 - 25%), certain characteristics are shared and the overall structures are likely similar. The shared features include four cysteine residues near the N-terminus, a tryptophan-serine-X-tryptophan-serine sequence (the WSXWS box, where X is any amino acid) near the C-terminus of the extracellular domain, and common patterns of hydrophobic and hydrophilic residues (Figure 2). Based on predictive structural analysis between the binding domains of the cytokine receptors and interferon receptors, the overall structure of the extracellular domain of this family of receptors was predicted to consist of two distinct immunoglobulin-like domains each containing seven β-strands (Bazan, 1990b).

Currently, the only three-dimensional structures available for members of this receptor family are that of the extracellular domain of the hGH receptor (hGHbp) complexed to hGH (de Vos et al., 1992) and the extracellular domain of the human prolactin receptor (hPRLbp) complexed to hGH (Somers et al., 1994). Although the sequence identity between the hGHbp and the hPRLbp is only 28%, the structures are very similar (Somers et al., 1994). The extracellular domain of each binding protein does, as predicted, contain two distinct immunoglobulin-like domains. The four cysteines which are conserved in the superfamily are buried in the core of the N-terminal β-sandwich of the hGHbp and the hPRLbp. The WSXWS box present near the C-terminus of the extracellular domain of all other members of the superfamily is not present in the hGHbp and is instead represented by the sequence tyrosine-glycine-glutamate-phenylalanine-serine (YGEFS) beginning at residue 222. In spite of the fact that the WSXWS box is highly conserved among these related receptors, the hGHbp structure indicates that this portion of the receptor is located away from the binding interfaces with hGH and does not appear to be involved in ligand binding or in the interactions between receptor molecules which are required for signal transduction. The WSXWS box in the hPRLbp, represented by the sequence WSAWS, is also quite removed from the ligand binding interface (Somers et al., 1994).
Although structures for other receptors in the hematopoietic superfamily are not yet available, the presence of conserved motifs within all members of the receptor family as well as the many structural similarities between the two reported extracellular domains suggests that the structure of the hGHbp can be used as a model for studying these related proteins as they interact with their ligands. Sequence alignments of receptors in the superfamily show similar patterns of hydrophobic and hydrogen-bonding residues, suggesting strong similarities in the folding and arrangement of the $\beta$-strands in the C-terminal region of the extracellular domain of the receptors. Thus, residues which are conserved may be anticipated to occupy equivalent positions in the different proteins, and the ligand-binding determinants of other receptors may correspond to those of the hGHbp. If this is indeed the case, then the residues of the WSXWS box, which had been predicted to form the "floor" of the binding crevice (Bazan, 1990) but which in the hGHbp and the hPRLbp are on a surface located away from the binding interfaces (de Vos et al., 1992; Somers et al., 1994) therefore may not be involved in ligand binding by any of the receptors. However, mutations in this region have been shown to disrupt ligand binding for the IL-2 (Miyazaki et al., 1991), erythropoietin (Chiba et al., 1992), and prolactin (Rozakis-Adcock and Kelly, 1992) receptors, suggesting that, at least for these proteins, this region plays an important role in the formation of the ligand binding site. It is also possible that this region may be required to bind a putative accessory protein (Wells and de Vos, 1993). Further study is needed to determine the role(s) of the WSXWS box in this receptor superfamily.

1.3.2 Intracellular Domains

Although the receptors in the hematopoietic superfamily exhibit homology in their extracellular ligand binding domains, the intracellular, cytoplasmic domains show considerable variation (Figure 2). These intracellular portions of the receptors vary greatly in length and sequence and the only region of homology, located near the membrane-spanning region, is a proline-rich motif (PRM) with the consensus sequence aliphatic-aromatic-proline-X-aliphatic-proline-X-proline (Al-Ar-P-X-Al-P-X-P) (O'Neal and Yu-Lee, 1993). It has been speculated that the PRM may be involved in binding to Src homology 3 (SH3) domains of downstream signaling molecules (Horseman and Yu-Lee, 1994).
In the case of the prolactin receptor (PRL-R), a number of different receptor forms have been identified. These include a short form of 291 amino acids containing a cytoplasmic domain of 57 amino acids, a long form of 592 residues with a 358 amino acid cytoplasmic domain, and an intermediate length receptor (393 amino acids) found in rat Nb2 lymphoma cells (Kelly et al., 1991, 1993). The different mRNAs which give rise to the short and long receptor forms are presumed to arise from alternative splicing of a single gene, while the Nb2 form of the receptor appears to be due to a mutation resulting in the loss of a significant portion of the cytoplasmic domain of the long form of the PRL-R (Kelly et al., 1989, 1991, 1993). These receptor isoforms have been shown to have tissue-specific expression and are also functionally different in terms of the biological effects which they mediate (Buck et al., 1992; Kelly et al., 1991; Lesueur et al., 1991). In addition, different receptor forms exhibit differences in hormone binding affinity: compared to the long form of the receptor, the intermediate length Nb2 PRL receptor showed a 3.3-fold increase in affinity for ovine PRL (oPRL). These results suggest that the intracellular domain of the receptor may influence hormone binding affinity (Ali et al., 1991).

Polymorphism of receptor structure has been noted for other members of the hematopoietin receptor superfamily including hGH, for which a soluble binding protein consisting of the extracellular domain of the receptor only has been identified (Baumann et al., 1986; Herington et al., 1986; Leung et al., 1987). Variation in receptor structures may therefore be a general feature of these related proteins (Kelly et al., 1991; Lesueur et al., 1991; Smith et al., 1988).

1.4 Signal Transduction

Elucidation of the signal transduction mechanisms involved in the actions of the receptors in the superfamily has been difficult because these proteins do not possess intrinsic enzymatic activity found in a variety of protein ligand receptors. A number of possible signal transduction pathways have been suggested for prolactin and growth hormone (reviewed in Kelly et al., 1991, 1993) and there is evidence that, although the growth hormone receptor does not show any homologies to known tyrosine kinases, cytoplasmic tyrosine kinase activity is associated with this receptor (Carter-Su et al., 1989). Many of these studies were carried out using Nb2 cells
which have become a paradigm for studying mitogenic signaling pathways of prolactin. Further investigations indicated that tyrosine kinase activation may be an early event associated with ligand binding to the prolactin receptor (Rui et al., 1992) and indeed, inhibition of tyrosine kinase activity was found to block the anabolic and mitogenic actions and thus, presumably, the signal transduction mechanism of lactogens (Carey and Liberti, 1993). Signaling from receptor to nucleus by prolactin appears to occur via cytoplasmic proteins associated with the prolactin receptor. Tyrosine phosphorylation of these associated proteins occurs following ligand binding to the receptor and this initiates a kinase cascade which leads to phosphorylation and activation of transcription factors. Once modified, these proteins bind enhancer elements present in the promoters of certain genes such as the interferon regulatory factor 1 (IRF-1) gene and the β-casein gene (David et al., 1994; Gilmour and Reich, 1994; Schmitt-Ney et al., 1991; Stevens and Yu-Lee, 1994).

Recent investigations have demonstrated that prolactin induces tyrosine phosphorylation and activation of the tyrosine kinase JAK2 (David et al., 1994; Rui et al., 1994a), suggesting that this enzyme is responsible for initiating the subsequent signal transduction events. A region of the prolactin receptor located proximal to the membrane-spanning region and comprising the proline-rich motif (PRM) has been identified as essential for interaction with JAK2 and subsequent signal transduction (DaSilva et al., 1994; Edery et al., 1994). However, although the association between JAK2 and the prolactin receptor appears to be very important for signal transduction, JAK2 is not the only kinase associated with the prolactin receptor. Another important kinase is the RAF-1 kinase: all forms of the PRL receptor were shown to be associated with the RAF-1 kinase in the PRL-dependent rat T-cell line Nb2, leading to phosphorylation and activation of RAF-1 (Clevenger et al., 1994). PRL stimulation of Nb2 cells was also found to activate the protein tyrosine kinase p59fyn, and revealed an association between the prolactin receptor and this src family kinase (Clevenger and Medaglia, 1994). Thus, both these kinases may act as intermediaries in the signaling pathway of PRL-mediated T lymphocyte proliferation.

JAK2 has also been shown to associate with the growth hormone receptor and to act as a signaling molecule for growth hormone (Argetsinger et al., 1993; Carter-Su et al., 1994). As is
the case for prolactin, the proline-rich region located near the transmembrane-spanning region of
the growth hormone receptor is required for association with JAK2 and for signal transduction
(Billestrup et al., 1994; Frank et al., 1994; Goujon et al., 1994; VanderKuur et al., 1994; Wang
and Wood, 1995). There is also evidence that JAK2 associates with the receptor for
erthropoietin and with other members of the receptor superfamily (Argetsinger et al., 1993;
Silvennoinen et al., 1993; Witthuhn et al., 1993) and is required for interferon-γ-activated gene
expression (Watling et al., 1993). Therefore, it seems likely that a common signaling cascade
mechanism involving JAK2 and/or other members of the JAK family exists for members of the
cytokine/hematopoietin superfamily. However, while the signaling mechanisms may be similar,
the marked differences in the physiological responses, as well as the variety of cell types
involved, suggest that there must be many parameters which regulate specific gene expression.
Further study is required before a complete understanding of the mechanism of action of these
hormones and cytokines is obtained.

1.5 Hormone-Receptor Interactions

A proposed mechanism of signal transduction for receptors with a single transmembrane
domain is that of ligand-induced receptor oligomerization (reviewed in Ullrich and Schlessinger,
1990). In brief, binding of the ligand induces receptor oligomerization which stabilizes
interactions between the cytoplasmic domains and activates the kinase functions which turn on
the signal transduction pathway. Investigations into the mechanism of hGH-receptor binding
have revealed that receptor oligomerization is involved in signal transduction in this system.
However, in contrast to the two ligand/two receptor stoichiometry which is commonly seen with
growth factor receptors, the hGH-hGHbp complex consists of one hGH molecule bound to two
receptor molecules (Ultsch et al., 1991).

Both physical and biochemical analyses have confirmed that hGH contains two receptor
sites (sites 1 and 2) which each bind to the same region of the hGHbp (Cunningham et al., 1991;
de Vos et al., 1992). Thus, each hGH molecule is bivalent while the hGHbp, which uses
essentially the same residues to bind to either site on hGH, is univalent. The hGH-(hGHbp)₂
complex forms sequentially: binding of a receptor molecule to site 2 only occurs following binding of a receptor to site 1. Thus, receptor binding at site 1 creates the binding site for the second receptor. Excess hGH can cause the hGH-(hGHbp)₂ complex to dissociate into one in which the hGHbp is bound exclusively at site 1 (Cunningham et al., 1991). Binding of two receptors to one molecule of hGH leads to receptor dimerization, a prerequisite for signal transduction: bivalent monoclonal antibodies (mAbs) to the hGHbp were as able as hGH to induce cell proliferation while monovalent Fab fragments had no effect. In addition, a hGH variant containing a mutation blocking receptor binding to site 2 was unable to activate proliferation (Fuh et al., 1992).

Receptor dimerization has also been demonstrated for the binding, in solution, of bGH to the extracellular domain of the bGH receptor, using gel filtration (Staten et al., 1993). The same study also showed that bovine placental lactogen (bPL), which is able to elicit both GH- and PRL-like responses in vivo by binding to bGH receptors, formed a 1:1 complex with bGHbp under the same experimental conditions. Although this result suggests the intriguing idea that very different mechanisms could be used to activate the same receptor, lack of evidence for dimerization of the extracellular portion of the receptor does not preclude the possibility of dimerization of the full-length receptor (Wells, 1994). As is the case for glycoporphin A (Lemmon et al., 1992), the transmembrane and/or intracellular portions of the growth hormone receptor could contain some of the determinants for dimerization.

Evidence has been obtained which suggests that dimerization is required to activate the prolactin receptors. Experiments with mAbs have shown that bivalent, but not monovalent anti-PRL receptor antibodies possess PRL-like activity (Elberg et al., 1990; Shiu et al., 1983). Furthermore, bivalent anti-PRLbp antibodies, with the concomitant dimerization of the PRL-R, were required for activation of JAK2 (Rui et al., 1994b). Monovalent anti-PRL-R Fab fragments were unable to dimerize the receptor and activate JAK2, but activity could be partially restored via cross-linking with bivalent anti-Fab antibodies. Binding of hGH to Nb2 PRL receptors has been shown to follow the two-site model of hGH-hGHbp interactions in that activity was seen with low concentrations of hGH while high concentrations resulted in inhibition of activity (Fuh...
et al., 1993). In addition, although the X-ray structure of hGH bound to the extracellular domain of the hPRL-R (hPRLbp) revealed a 1:1 association between these proteins, comparisons between this structure and the structure of the hGH-(hGHbp)$_2$ led to the suggestion that an intermediate form ready to bind a second hPRLbp and form an active complex was captured in the crystal hGH-hPRLbp complex (Somers et al., 1994). However, conflicting results have been obtained with respect to the interaction of PRL with the PRL-R. One group using the extracellular domain of the rabbit PRL-R did not observe a 1:2 complex of hormone with receptor (Gertler et al., 1993), while another group using the extracellular domain of the rat liver PRL-R obtained data supporting the model of dimerization of the PRLbp by one PRL molecule (Hooper et al., 1993). A mutational analysis of hPRL binding to the hPRLbp revealed that while alanine replacements of residues in the putative site 2 binding site of hPRL did not diminish the mitogenic activity of the hPRL variants, replacements of some of these residues (A22, L25, and G129) with tryptophan or arginine resulted in variants with bioactivities in the Nb2 cell assay two to three orders of magnitude less than hPRL (Goffin et al., 1994). These results thus suggested the existence of a second binding site on hPRL for the Nb2 PRL receptor. Definitive proof of whether PRL forms a complex with two molecules of PRL-R will require a determination of the crystal structure of the complex; however, the evidence so far collected generally supports this two-site model.

1.6 Mutational Analysis of Hormone Binding to Receptors

A complete understanding of the interactions between a ligand and its receptor requires complementary information obtained from mutational mapping of the binding sites and from the three-dimensional structure of the ligand-receptor complex. Although three-dimensional structures are available for only a few members of the hematopoietic superfamily, a number of mutational analyses have been carried out and these have provided a wealth of information regarding the interactions of these proteins with their receptors.
1.6.1 Human Growth Hormone

A consequence of the many studies which have focussed on examining the interactions of hGH with its receptor is that this hormone is now one of the best characterized members of the hematopoietic superfamily. Human growth hormone is a 191 amino acid globular protein containing two disulfide bridges, four α-helices in the core of the molecule, and an additional three short helical segments in the connecting loops between the helices (de Vos et al., 1992; Figure 3). Naturally occurring growth hormone is a very heterogeneous polypeptide hormone (Baumann, 1991), and prior to the development of efficient bacterial expression systems for hGH (Chang et al., 1987; Hsiung et al., 1986) and the concomitant increased ease of performing site-directed mutagenesis studies, hormone-receptor interactions were investigated using natural and chemically-generated hormone variants and peptide fragments (Aubert et al., 1986; Chawla et al., 1983; Chêne et al., 1989; Kostyo, 1974; Lewis, 1984). These studies were unable to provide a clear understanding of which portions of the hGH molecule are responsible for biological activity; however, they did indicate possible general areas of biological importance. The application of molecular biology techniques such as site-directed mutagenesis to this problem have since allowed a thorough analysis of the interactions of this hormone with its receptor.

**Figure 3.** Diagram of hGH showing its two disulfide bridges and the locations of the helical regions. Numbers indicate the position of the cysteine residues and the limits of the helical regions. Labels indicate the four α-helices which form the core of the three-dimensional hormone molecule. The short helical sections in the loops connecting the helices (residues 38 - 47, 64 - 70, and 94 - 100) are indicated only as hatched boxes.
1.6.1.1 hGH Fragments, Variants, and Chimeric Proteins

Analysis of the growth promoting activity of recombined peptide fragments of bovine GH, namely residues 1 - 95 linked by a disulfide bond to 151 - 191, residues 96 - 133, and residues 134 - 150, obtained from a tryptic digest of hormone isolated from pituitary extracts, indicated that residues 134 - 150 of the hormone were not necessary for biological activity (Hara et al., 1978). The N-terminal 134 amino acid section of hGH, obtained from a plasmin digest of a preparation of pituitary hGH, was also sufficient to promote biological activity, although full expression of activity required the entire molecule (Reagan et al., 1975). Recombinant proteins obtained through the noncovalent interaction of the N-terminal 134 amino acid portion of hGH with parts or the whole of the C-terminal portion (Li et al., 1976; Li and Blake, 1979; Reagan et al., 1981) exhibited nearly full biological activity. These results suggested that the disulfide loop joining C53 and C164 is not necessary for activity.

A number of studies of the N-terminal two-thirds of the molecule suggested a more restricted localization of growth-promoting activity to several different peptides, including residues 15 - 125 and 96 - 124 (reviewed in Chêne et al., 1989). A fragment consisting of residues 96 - 133, containing helix 3 of growth hormone (amino acids 109 - 126), was isolated from tryptic digests of bovine growth hormone (bGH) and had growth promoting activity (Gráf and Li, 1974; Gráf et al., 1976; Yamasaki et al., 1970). Recently, the role of helix 3 has been investigated further through the synthesis of several peptides corresponding to residues in this region. No stimulation of Nb2 cell growth, as normally observed with the presence of primate GH's, was seen with a peptide with the same amino acid sequence as that from residues 110 - 127 of hGH; however, replacement of E117 with leucine enhanced the amphipathic nature of the helix and stimulated mitogenesis (Kornberg and Liberti, 1992). Although intriguing, these results contradict findings from mutagenesis studies (discussed in Section 1.6.1.2) and have not been followed up. Another peptide encompassing residues 108 - 129 of hGH elicited a mitogenic response in a system using preadipocytes wherein native hGH is anti-mitogenic, but its actions appeared to be mediated through binding to a site other than the growth hormone receptor (Jeoung et al., 1993). Unfortunately, while these results suggest that helix 3 may play
an important role in signal transduction they do not provide a clear picture of the region's contribution to the biological activity of hGH.

In general, whilst suggestive, the use of peptide fragments to identify regions of growth hormone important for biological activity, or to determine the contribution of particular segments to activity, has not been very informative. Although these studies led to the idea that growth hormone may contain several "active sites," the weak receptor binding typically displayed by peptide fragments and the possibility that differences in activity between the fragments and the complete hormone could simply reflect differences in structure prevent a thorough dissection of hormone structure as related to function.

The use of natural variants of the hormones is one way to get around the potential problems of peptide fragments. Among the natural variants of hGH examined is a 20,000 Da protein lacking the 15 amino acids between residues 32 - 46 (Lewis et al., 1980). The growth-stimulating abilities and lactogenic effects of this variant were found to equal those of full-length hGH; however, a marked decrease in the insulin-like activity suggested that residues 32 - 46 may be involved in this activity (Chêne et al., 1989). Although the 20 kDa variant was found to be as effective as wild-type hGH in causing dimerization of the hGHbp (Cunningham et al., 1991), differences in affinities between the two forms of hGH in binding to receptors have been reported and these may give rise to the variations in biological effects (Beattie, 1993). In any case, residues 32 - 46 of hGH appear to define a significant but less critical part of the binding domain of hGH (Sigel et al., 1981; Wohnlich and Moore, 1982).

Other attempts to relate structural features to biological properties focussed on sequence comparisons between related hormones (Nicoll et al., 1986). These results implicated certain clusters of residues as being potential components of a hormone-specific binding domain; however, these conclusions also exhibited a number of inconsistencies, including identification of putative prolactin-specific clusters that were not found for other lactogenic hormones such as hGH and human placental lactogen (hPL). One drawback to using sequence comparisons to delineate regions of functional importance is that the multiple amino acid variation between
hormone sequences means that the contribution of specific residues to hormone function cannot be determined.

Chimeric hormones, created by combining fragments from related hormones, have allowed the contribution of specific portions of the hormones to biological activity to be examined while avoiding severe disruptions to the protein structure. Recombinant hormones containing fragments of hGH and hPL in which the N-terminal fragment (amino acids 1 - 134) of each was combined with the C-terminal fragment (residues 141 - 191) of the other, prepared by the covalent joining of peptide fragments generated by limited plasmin digestion and reduction, revealed that the biological activities of each hormone resided in the N-terminal fragment. Thus, the chimeric hormone consisting of the N-terminal portion of hGH fused to the C-terminal portion of hPL (hGH-hPL) exhibited activities similar to native hGH, while the hPL-hGH chimeric hormone had lactogenic activity only (Russell et al., 1981). The N-terminal section therefore appeared to contain binding site(s) responsible for specificity while the C-terminal was involved in stabilizing the active conformation of the hormone.

The affinity towards lactogenic receptors of a chimeric hormone consisting of residues 1 - 23 of the nonlactogenic bGH joined to residues 24 - 191 of hGH, prepared by recombinant DNA technology and produced in E. coli, was found to be several orders of magnitude lower than that of hGH alone while the affinity towards somatogenic receptors was decreased to a much lesser degree. This result indicated that the binding site of hGH with high affinity for lactogenic receptors includes residues in the N-terminal 23 amino acid portion of the hormone (Binder et al., 1989). Replacement of the N-terminal 13 amino acids of hGH by the corresponding bGH sequence had only a slight effect on binding to lactogenic receptors while binding to somatogenic receptors was reduced to a higher degree (Binder et al., 1990). In earlier studies the biological activity of hGH molecules lacking the N-terminal 7 or 13 residues, created by site-directed mutagenesis, was examined and it was found that, compared to full-length hGH, the molecule lacking 13 amino acids had reduced affinity towards the lactogenic receptors of Nb2 lymphoma cells while the loss of the N-terminal 7 residues had no effect (Ashkenazi et al., 1987; Gertler et al., 1986). After taking all these results into account, along with data from an attempt
to identify functionally important domains through hydropathy plots (Nicoll et al., 1986) the investigators suggested that the high-affinity hGH binding site for lactogenic receptors may be located between residues 8 and 18 (Binder et al., 1989). Similar conclusions were reached following a study in which high affinity mAbs with a defined epitope specificity profile against hGH were used to inhibit $^{125}$I-hGH binding to lactogen and somatogen receptors (Strasburger et al., 1989). Monoclonal antibodies that affected binding to one type of receptor did not necessarily affect binding to the other. These researchers suggested that the lactogen receptor binding site is mainly confined to the N-terminal portion of hGH, particularly residues 8 - 13, while sections of the N-terminal and C-terminal are necessary for binding to the somatogen receptor.

Further investigations into the biological activities of the hGH mutant lacking the first 7 amino acids revealed that, although this hormone retained full ability to bind to lactogenic receptors, it exhibited a markedly reduced ability to bind to somatogenic receptors and was also unable to promote diabetogenic or insulin-like activity. Therefore, the initial 7 residues at the N-terminal end of hGH appear to be required for expression of the growth promoting activity (assessed on the basis of weight gain in hypophysectomized rats), diabetogenic activity (determined with respect to glucose tolerance in ob/ob mice), and insulin-like activity (measured as the ability to stimulate $^{14}$C-glucose oxidation by epididymal adipose tissue of hypophysectomized rats) (Towns et al., 1992). These effects are presumably mediated by the involvement of this portion of hGH in somatogenic receptor dimerization: removal of the first 8 residues of hGH has been noted to interfere with binding at site 2 and therefore prevent hGHbp dimerization (Cunningham et al., 1991). However, since removal of the first 7 residues did not decrease affinity for the Nb2 lactogenic receptors while removal of the first 13 residues did (Ashkenazi et al., 1987; Gertler et al., 1986), dimerization of the lactogenic receptors by hGH may involve slightly different residues than those necessary for dimerization of the somatogenic receptors.

While the conclusions from the various studies discussed in this section do not distinguish the specific residues involved in the interaction of hGH with somatogenic and lactogenic
receptors they do identify regions of the hormone that are critical for activity. Thus, early investigations, analyzing peptide fragments, localized biological activity to the N-terminal portion of hGH since the first 134 amino acids were sufficient to promote most of the function of the hormone. Correlation of various activities of hGH to discrete sequences within this region came from later studies analyzing chimeric hormones. Specifically, residues 32 - 46 were found to be required for insulin-like activity, amino acids 8 - 18 were necessary for lactogenic activity, and the first 7 residues were important for somatogenic activity. In addition, although the evidence was not clear-cut, there was an indication that residues within helix 3 of hGH (amino acids 109 - 126) may be involved in signal transduction.

1.6.1.2 Site-Specific Mutants of hGH

A high resolution functional analysis of hGH-receptor binding has followed the development of efficient bacterial expression systems for recombinant hGH (Changeta!., 1987; Hsiung et al., 1986), the determination of a structural model for pGH (Abdel-Meguid et al., 1987), the cloning of the hGHbp (Leung et al., 1987), and efficient bacterial production of this extracellular domain of the hGH receptor (Fuh et al., 1990). The ability to easily produce the extracellular portion of the receptor led to the development of an assay wherein the contribution of specific hGH residues in binding to the hGHbp, and thus presumably to the activity of the hormone, was determined with respect to whether these side chains strongly modulated binding to the receptor. In contrast, the activities of the variants and chimeras discussed in the previous section were determined by a number of bioassays: loss of bioactivity was presumed to relate to disruption of binding interactions between the hormone and various receptors.

Regions of the hormone involved in receptor binding were initially mapped using a strategy called homolog-scanning mutagenesis (Cunningham et al., 1989). Using the model of pGH (Abdel-Meguid et al., 1987) as a guide, a set of chimeric hormones that collectively modified 85 out of 191 residues in hGH was created by substituting segments (7 to 30 residues) of hGH with corresponding regions from homologous hormones that do not bind to the hGHbp (namely hPL, hPRL, and pGH). Sequences chosen for substitution were within borders of secondary structure and were thus limited to specific putative helical or loop regions. In
addition, alteration of residues on the hydrophobic faces of the helices was avoided since mutations of buried residues in hydrophobic cores are generally destabilizing (Alber et al., 1987; Pakula et al., 1986). It was found that the segment substitutions which affected binding of certain of the chimeric proteins to the hGHbp were from three discontinuous regions of the hormone: the N-terminal section of the first helix, a portion of the loop connecting helices 1 and 2, and the C-terminal section of the fourth helix. When these segments were mapped on the three-dimensional model of the hormone it was apparent that although discontinuous in terms of primary structure, they are continuous with respect to tertiary structure in that they form a single patch (Cunningham et al., 1989).

The specific residues that modulate binding to the hGHbp were identified using a technique called alanine-scanning mutagenesis (Cunningham and Wells, 1989). In this technique many different mutants are prepared, each containing a single alanine substitution of a residue within the region of interest. Although analysis of these variants can identify which residues are involved in function, the results can also be misleading since it is possible for nearby side chains in the three-dimensional structure of the protein to substitute for the residue replaced by alanine. Therefore, a lack of loss of activity following an alanine replacement does not guarantee that that specific residue is not involved in the function of the protein. In the case of hGH, each individual residue within the three segments already implicated in receptor binding was mutated to alanine, the mutant proteins were expressed in a secreted form from E. coli, and the binding constant to the extracellular domain of the hGH-R was determined for each mutant by competitive displacement of $^{125}$I-labeled hGH. Within helix 1, alanine substitutions at residues 6, 10, and 14, all located on the same face of the helix, had the most disruptive effect on binding. In addition, residues 54, 56, 58, 64, and 68 of the loop region joining helices 1 and 2 and residues 171, 172, 174, 175, 176, 178, 182, and 185 of the C-terminal portion of helix 4 were also identified as being involved in binding to the receptor (Site 1 in Figure 4). In general, the side chains of these various residues extend from the same side of hGH and together form the discontinuous binding site first identified through homolog-scanning mutagenesis. The inability
of peptide fragments to mimic this discontinuous binding site explains why earlier studies using peptides were not able to precisely define this region.

The data obtained from the mutagenesis studies support a number of earlier investigations in which chemical modification was used to determine the contribution of specific residues to receptor binding. Ethoxyformylation of H19 and/or H21 (both in helix 1) interfered with the receptor binding ability of equine GH (Fukushima et al., 1987). Covalent labeling of K70 of hGH (located in the loop between helices 1 and 2) with fluorescein isothiocyanate resulted in a hormone variant with decreased mitogenic ability in the Nb2 cell bioassay and lowered affinity towards somatogen receptors in bovine liver and towards lactogen receptors in Nb2 cells (Sakal et al., 1991). In addition, acetylation of K167 or K171 (both located in helix 4) with 3H-acetic anhydride disrupted binding of hGH to rat liver somatogenic receptors (Teh and Chapman, 1988).

![Structural model of hGH indicating the location of sites 1 and 2 for binding to the hGHbp.](image)

**Figure 4.** Structural model of hGH indicating the location of sites 1 and 2 for binding to the hGHbp. Residues where alanine substitutions reduced binding to the extracellular domain of the receptor are indicated by closed circles (for site 1) and squares (for site 2), with increased symbol size correlating with increased reduction in binding affinity. The open circle indicates that the variant E174A displayed an increase in binding affinity for the hGHbp. The site 1 residues indicated were identified as those in which alanine mutations caused a fourfold or greater change in the dissociation constant for the soluble portion of the receptor. Site 2 residues were identified using a fluorescence quenching dimerization assay wherein mutants were examined for their ability to disrupt receptor dimerization without affecting binding to site 1. The figure is taken from Cunningham et al., 1991.
As discussed above, the mutagenic analysis of the interaction between hGH and the hGHbp identified three regions of the hormone that form the binding domain for the receptor: the N-terminus of helix 1; the loop between helices 1 and 2; and the C-terminus of helix 4 (Cunningham and Wells, 1989). The functionally important residues in these regions form a single binding site, designated Site 1 in Figure 4. These results were obtained prior to the discovery that a single hGH molecule binds two receptor molecules. Indeed, the second site was not identified in these early studies because the monoclonal antibody used to detect receptor binding blocked binding of a second hGHbp to hGH and thus only allowed formation of a monomeric complex (Cunningham et al., 1991). Receptor dimerization was demonstrated using a sensitive solution assay in which quenching of the fluorescence from a fluorescein-labelled hGHbp was measured following the addition of hGH: the addition of 0.5 M equivalents of hGH caused maximal fluorescence quenching (Cunningham et al., 1991). Using this assay a number of hGH variants, including deletion mutants, chimeric proteins of hGH with hPL or hPRL segment substitutions, and alanine-substitution mutants were examined for their ability to reduce receptor dimerization without affecting site 1 binding. Those residues for which substitutions were the most disruptive were mapped to the N-terminus and to the centre of helix 3 (Site 2 in Figure 4) (Cunningham et al., 1991). These results supported an earlier study wherein exon exchange between the hGH gene and the rat PRL or rat GH genes revealed that the region encoded by exon 4, which includes helix 3, is required for somatogen receptor binding (Ray et al., 1990) and were confirmed by the crystal structure of the hGH-(hGHbp)2 complex which indicated that residues in this region form binding determinants (de Vos et al., 1992).

The importance of helix 3 residues (amino acids 109 - 126 in hGH) to receptor binding and biological activity has been confirmed by several other studies. Transgenic mice expressing hGH analogs containing the mutation G12OR exhibited a growth-suppressed phenotype, with the degree of growth suppression being directly correlated with the serum levels of G12OR. On the other hand, an enhanced growth phenotype was noted with mice expressing a G120A mutation (Chen et al., 1994). Studies on bGH indicated the same involvement of helix 3 in biological activity as was shown for hGH (Chen et al., 1991a,b, 1995). Human GH molecules containing
mutations in helix 3 which disrupt site 2 binding retain a functional site 1 and are able to bind to the hGHbp. However, the G120R mutation, by sterically blocking site 2, prevents receptor dimerization and thus this variant acts as a hGH antagonist (Fuh et al., 1992).

As listed above, the various mutagenic analyses of the interaction of hGH with the hGHbp have identified a number of residues that contribute to the binding of the hormone to the receptor. However, although alanine scanning mutagenesis identified approximately 30 side chains as being involved in binding, an analysis of the energetic importance of these side chains indicated that approximately 85% of the binding energy is accounted for by only 8 of these residues (Cunningham and Wells, 1993). Thus, the functional binding site of hGH, consisting of those residues which when substituted with alanine caused a greater than twofold reduction in binding affinity, is much smaller than the structural binding site, which is composed of the residues at the contact interface between the hormone and receptor. Some of the structural contacts identified by X-ray crystallography are functionally silent or even deleterious as measured by alanine scanning mutagenesis. These residues include H18, H21, R167, K168, and E174 in site 1 and N12, R16, R19 and N109 in site 2. It is not yet clear why contacts between the side chains of these residues and the receptor may be functionally silent but it is possible that the energy required to desolvate these charged and hydrogen-bonding groups may not be offset by the free energy gained from binding (Cunningham and Wells, 1993).

From the crystal structure of the growth hormone-receptor complex it can be seen that most of the side chains comprising the functional binding site do contact the receptor (de Vos et al., 1992). The remaining residues identified by the mutagenesis studies, including F10, F54, I58, and F176, are buried in hydrophobic clusters and substitutions of these may disrupt the binding of contact residues via structural changes (Wells and de Vos, 1993). An analysis of the contributions of residues in the structural binding site of the hGHbp to the binding affinity similarly revealed that only a few of these residues, those mapping to the centre of the region, form the functional binding site (Clackson and Wells, 1995). When examined in the context of the hGH-(hGHbp)$_2$ complex, the functional binding sites of hGH and the hGHbp join together to form a tightly packed hydrophobic core surrounded by hydrogen bonds and five intermolecular
salt bridges. Thus, only a few interactions seem to be important for tight binding. However, the residues identified in the structural but not the functional binding site may play important roles such as increasing the rate of hormone-receptor association or contributing to the specificity of binding (Clackson and Wells, 1995). Indeed, as discussed in the following section, the location of these functionally "null" contact residues is in the region important for binding hGH to the hPRLbp (Cunningham and Wells, 1991).

1.6.1.3 hGH Interactions with PRL Receptors

There are many similarities between growth hormones, prolactins, and placental lactogens, but in general the functions and binding specificities of each are distinct. Among the few exceptions to this are growth hormones from primates which, in addition to having growth hormone activities, exhibit lactogenic activity as a result of binding to lactogenic receptors. However, as discussed in section 1.6.1.1, there is much evidence to suggest that different residues of the hGH molecule are responsible for the somatogenic and lactogenic activities (Ashkenazi et al., 1987; Binder et al., 1989, 1990; Gertler et al., 1986; Strasburger et al., 1989; Towns et al., 1992). This was also suggested by the observation that chimeric hormones created by switching exons 3 or 4 of the hGH gene with the corresponding exons of the rat PRL or rat GH genes retained some degree of lactogen receptor binding while losing the ability to bind to somatogen receptors (Ray et al., 1990). The differences between the somatogen and lactogen receptor binding determinants were confirmed by a mutagenic analysis of the interaction of hGH with the hPRLbp which indicated that the binding determinants on hGH for the growth hormone and prolactin receptors overlap but are not identical (Cunningham and Wells, 1991). Alanine-scanning mutagenesis identified a number of hGH residues which, when mutated to alanine, caused a greater than fourfold reduction in binding affinity to the prolactin receptor. These residues are found in the central section of helix 1 (H18, H21, and F25), in the loop region between helices 1 and 2 (I58, N63, and S62), and in the middle of helix 4 (R167, K168, K172, E174, F176, and R178). When mapped onto the structural model of hGH, the patch formed by these residues is similar to that formed by residues comprising the binding site for the hGHbp, but is shifted towards the centre of helices 1 and 4 and does not include the N-terminus of helix 1.
or the extreme C-terminus of helix 4 (Cunningham and Wells, 1991) (Figure 5). Within this patch are residues M64 and M179: oxidation of either one or both of these amino acids resulted in a marked reduction in affinity of hGH and hPL for lactogenic receptors (Teh et al., 1987).

The differences between the binding determinants of hGH for the growth hormone and prolactin receptors are great enough that receptor-specific variants were created by mutating residues identified as being essential for binding to one receptor but not the other (Table 1). Thus, the double mutant resulting from a combination of the mutations K168A and E174A, which each preferentially disrupt binding to the hPRLbp, exhibited a 34,000-fold shift in preference for binding to the hGHbp. Similarly, binding to the hPRLbp was enhanced nearly 150-fold by combining the mutations R64A and D171A, each of which disrupt binding to the hGHbp. Binding to both receptors was deleteriously affected by combining mutations at residues such as K172 and F176 which are important for binding of hGH to both the hPRLbp and the hGHbp.

Table 1. Binding of Double Mutants of hGH to the hPRL and hGH Binding Proteins (hPRLbp and hGHbp)*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>hPRLbp $K_d$(mut)/$K_d$(hGH)</th>
<th>hGHbp $K_d$(mut)/$K_d$(hGH)</th>
<th>Change in receptor preference: $hPRLbp/hGHbp$</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGH</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>K168A/E174A</td>
<td>9100</td>
<td>0.27</td>
<td>34000</td>
</tr>
<tr>
<td>R64A/D171A</td>
<td>1.9</td>
<td>280</td>
<td>0.0068</td>
</tr>
<tr>
<td>K172A/F176A</td>
<td>8400</td>
<td>560</td>
<td>15</td>
</tr>
</tbody>
</table>

* Data are taken from Cunningham and Wells, 1991.

The dissociation constants for variants of hGH to the hGHbp, [$K_d$(mut)], were measured by competitive displacement of $^{125}$I-labeled hGH from the hGHbp. A monoclonal antibody to the hGHbp was used to precipitate the $^{125}$I-hGH-hGHbp complex. The ratio $K_d$(mut)/$K_d$(hGH) indicates the relative reduction in binding affinity of the hGH variants to that of wild-type hGH for the hGHbp.

The change in receptor preference was calculated by dividing the ratio of the relative reduction in binding affinity for the hPRLbp by that for the hGHbp.
A major difference between hGH binding to the growth hormone and prolactin receptors was revealed in an earlier study which indicated that Zn$^{2+}$ is required for binding of hGH to the hPRLbp (Cunningham and Wells, 1990a) (Figure 5). Zn$^{2+}$ is not required for formation of the hGH-(hGHbp)$_2$ complex or for binding of hPRL to the hPRLbp. The ligands identified in mediating binding of hGH to the hPRLbp in the presence of Zn$^{2+}$ are H18 and E174 which, in conjunction with two ligands on the receptor (D217 and H218) form a Zn$^{2+}$-binding site (Somers et al., 1994). Initially, it was surmised that H21 is also involved in binding Zn$^{2+}$ (Cunningham et al., 1990a); however, crystallographic analysis revealed that this residue is not a direct ligand to the Zn$^{2+}$ but is instead required to correctly orient the side chain of E174 (Somers et al., 1994).

The possible importance of residues within the loop region (residues 54 - 74) for PRL receptor-binding specificity was investigated by a comparison of the sequence of the lactogenic and somatogenic hGH with the somatogenic but non-lactogenic bGH: residues 57 and 60 differ in these hormones. T57 and A60 of bGH were changed to the corresponding hGH residues (serine and threonine, respectively) in order to determine if these residues are important for the lactogenic activities of hGH (Chen et al., 1991c). In fact, the PRL receptor-binding properties of the variants were unchanged from that of wild-type bGH, suggesting that these residues do not determine the receptor-binding properties. More recently, another mutagenic analysis of residues within the 54 - 74 loop of hGH was carried out in order to investigate the involvement of these residues in lactogenic and somatogenic function (Sakal et al., 1993). The affinity of the P61A mutant for the hGHbp was 12.5% that of the wild-type hGH and the affinity of a double mutant P59A/P61A was 7%. In both instances the variant hormones formed 1:2 complexes with the receptor. However, while these mutations affected binding to the hGHbp, the ability of these variants to stimulate proliferation of Nb2 cells was unchanged from that of unmodified hGH. These results suggest that different amino acids in the residue 54 - 74 loop region are responsible for interacting with somatogenic and lactogenic receptors or that this region is not involved in lactogenic receptor activation.

The X-ray structure of a hGH molecule complexed to an hPRLbp (Somers et al., 1994) showed that although essentially the same hormone and receptor residues form the hormone-
receptor interfaces for both the hGH-hGHbp and hGH-hPRLbp complexes, the orientations of the N- and C-terminal domains of the receptors are not identical. Since each hormone molecule binds two receptor molecules, this difference in domain orientation may be important in order to prevent the formation of mixed complexes wherein the hormone is bound to both a hGH-R and a hPRL-R.

Figure 5. Structural model of hGH indicating the binding site for the hPRLbp. Residues where alanine substitutions caused a reduction in binding affinity are indicated by closed, graduated circles, with increased symbol size correlating with increased reduction in binding affinity. The dotted lines joining residues H18 and E174 to the Zn\(^{2+}\) molecule indicate the role that these amino acids play in forming part of the binding determinant for Zn\(^{2+}\). Zn\(^{2+}\) is required for binding hGH to the hPRLbp but not for binding hPRL to the hPRLbp. Figure is taken from Cunningham and Wells, 1991.

1.6.2 Placental Lactogen

Very few studies have been carried out to investigate which residues of placental lactogen are involved in receptor binding. Indeed, very little is known in general about this member of the hormone family. Placental lactogen, in common with other lactogens, binds to the PRL-R but a specific PL-R also has been identified (Freemark and Comer, 1989). Detailed analyses of PL-receptor binding have not yet been carried out. However, the data that have been obtained from a few structure-function analyses of placental lactogen have provided insight into the receptor-
mediated activities of this hormone and have reinforced the concept that the receptor-binding determinants of members of this hormone family are very similar.

A comparison of the sequences of six related mouse proteins, three lactogenic (mPRL, mPL-I, and mPL-II), and three non-lactogenic [mGH, proliferin (mPLF), and proliferin-related protein (mPRP)], identified five residues that are conserved among the lactogenic hormones but are different in the nonlactogenic hormones (Davis and Linzer, 1989b). The locations of these residues (R14 near the N-terminus of helix 1 and R165, R169, K179, and N189 in the C-terminal half of helix 4) are in regions which in the related protein hGH are important for hGH-hGHbp interactions (Cunningham and Wells, 1989) and it therefore seemed likely that these mPL-II residues might be involved in binding to the prolactin receptor. A mutagenic analysis of these residues revealed that mPL-II variants with mutations at R14 (in putative helix 1), R169, or K179 (in putative helix 4) were unable to stimulate the growth of Nb2 cells or bind to the hepatic PRL receptor (Davis and Linzer, 1989b). Thus, these residues appear to be critically important for hormone interaction with the lactogenic receptor. R165 and N189 were not, in themselves, absolutely required for receptor-binding; however, a combination of mutations at either of these positions with mutations at R169 or K179 caused a further decrease in the ability of these proteins to stimulate Nb2 cell growth. The involvement of helices 1 and 4 in binding of mPL-II to the mPRL receptor was further indicated by the inability of chimeric proteins, in which helix 1 or 4 of mPL-II were replaced with the corresponding regions of the non-lactogenic mPLF, to bind to the receptor.

Another mutational analysis of mPL-II focussed on determining what, if any, role the conserved cysteine residues play in receptor binding and hormone function (Davis and Linzer, 1989a). Placental lactogen, like growth hormone, contains two disulfide bonds: one connecting the loop region between helices 1 and 2 with helix 4 (C51 and C166 in mPL-II); and one at the C-terminus (C183 and C191 in mPL-II). These cysteines are conserved throughout the growth hormone/prolactin/placental lactogen family of hormones. Disruption of either disulfide bond through the mutation C51S or C183S did not lead to a change in the affinity for the PRL receptor. However, while the mitogenic activity of the C183S mutant was unaffected, the C51S
mutant was unable to stimulate the growth of Nb2 cells. Therefore, disruption of the C51 - C183 disulfide bond and/or mutation of residue C51 resulted in a variant hormone able to bind the PRL receptor but not leading to receptor-mediated mitogenesis. In order to cause this effect the C51S variant may interfere in some manner with receptor dimerization, but the exact nature of the interaction of placental lactogen, or of this variant, with the prolactin receptor is as yet unknown.

Bovine placental lactogen differs from that of other species including human and mouse in that this hormone has an additional 13 N-terminal amino acids. A third disulfide bond is contained in this region, a feature commonly found in prolactins from mammalian species. The importance of the N-terminal region, including the N-terminal "nose" as well as helix 1 (postulated to begin at residue 19), to receptor-binding and subsequent signal transduction was investigated through the creation and analysis of a number of N-terminus-truncated bPL variants (Gertler et al., 1992). Removal of the first 15 residues caused an approximately 50% decrease in stimulation of Nb2 cells but resulted in an increase in somatogenic receptor-mediated 3T3-F442A preadipocyte bioactivity. Further truncations by removal of up to 20 residues essentially abolished lactogenic activity and also decreased somatogenic activities, albeit not to the same extent as the lactogenic actions. It was thus suggested that the N-terminal "nose" of bPL may be required for binding to the PRL receptors but not for the interaction between bPL and somatogenic or unique bPL receptors (Gertler et al., 1992). However, while this may be the case for bPL, placental lactogens of other species lack this N-terminal 13 amino acid sequence but are nonetheless able to bind to the prolactin receptor and cause receptor-mediated mitogenesis (Davis and Linzer, 1989b; Deb et al., 1989; Lowman et al., 1991).

While the affinities of hPL and hGH for the hPRLbp are almost identical (in the presence of 50 μM ZnCl₂) hPL binds very weakly to the hGHbp, even though hPL shares 85% sequence identity with hGH (as opposed to 23% with hPRL) (Lowman et al., 1991). This difference in binding affinities is not unique: mammalian growth hormone receptors have high affinity for mammalian growth hormones and sheep or goat placental lactogens but low affinity for mammalian prolactins and primate placental lactogens; and lactogenic receptors bind lactogenic hormones but have low affinity for somatogenic hormones (Freemark and Corner, 1989; Lesniak
The similarity in binding affinities of hGH and hPL for the hPRL receptor appears to be a consequence of the similarity in critical amino acid sequence in these hormones for the hPRLbp (Lowman et al., 1991): the only difference in the side chains involved in binding to the hPRLbp is residue 25 (phenylalanine in hGH, isoleucine in hPL). The affinity of hPL for the hGHbp is 2,300-fold less than that of hGH for the hGHbp; however, by altering residues within the receptor-binding site the specificity of hPL for the hPRL and hGH receptors could be selectively changed (Lowman et al., 1991). Thus, substitution of four residues from hGH into hPL (V41I, D56E, M64R, and M179I) raised the affinity to the hGHbp from 2,300-fold weaker than hGH to only 11-fold weaker. The binding affinity of this variant for the hPRLbp was only slightly reduced. However, substitution of E174, involved in coordinating Zn\(^{2+}\) in both hPL and hGH, by alanine decreased the affinity for the hPRLbp by 1,400-fold. The combination of this mutation with those listed above increased the affinity of hPL for the hGHbp to only 6-fold less than hGH. One additional mutation, M64K, shown previously to enhance hGH binding to the hGHbp (Cunningham et al., 1990b), increased hPL affinity to 1.6-fold less than hGH.

1.6.3 Prolactin

As described above, the members of the growth hormone/prolactin/placental lactogen superfamily of hormones have many similarities with respect to receptor interactions, although subtle but important differences have been noted. Investigations have been carried out to define the structure-function relationships of prolactin, and these have confirmed the resemblance to growth hormone and placental lactogen. However, much work remains to be done before a complete understanding of how prolactin elicits all of its various physiological effects is obtained.

1.6.3.1 Prolactin Variants

Prolactin is a heterogeneous hormone in that it undergoes a number of different types of post-translational modifications including glycosylation, phosphorylation, deamidation, aggregation, and proteolysis. Prolactin also plays a role in regulating many different biological processes; however, whether the different PRL variants mediate different biological functions is
unknown (see Cole et al., 1991 and Smith and Norman, 1990 for reviews of variants and activities).

One of the prolactin variants which has been studied in terms of its ability to give rise to PRL-like effects is a 16 kDa N-terminal fragment, generated by proteolytic cleavage followed by reduction of the disulfide bonds. Obtained initially from rat and then from human, this fragment was reported to act as a mitogen for Nb2 cells, albeit at a lower activity than full-length rPRL (Clapp et al., 1988). However, since this fragment was obtained by in vitro cleavage of full-length rPRL it is possible that some of the observed bioactivity may have been a result of contamination with native 23 kDa rPRL. In support of this, independent studies showed no activity of 16 kDa fragments of recombinant hPRL in the Nb2 cell assay (Dr. Goffin, Inserm Unite 344, Endocrinologie Moleculaire, Paris, France, personal communication). An investigation into the binding characteristics of the 16 kDa rPRL fragment provided evidence for specific 16 kDa rPRL-binding sites, particularly in brain and kidney membranes (Clapp et al., 1989). Further research showed that the rat and human 16 kDa fragments, but not full-length rPRL or hPRL inhibited in vitro angiogenesis, that is, the regulation of new blood vessel formation (Clapp et al., 1993; Ferrara et al., 1991). It was suggested that this effect is mediated through a receptor specific for the cleaved form of PRL (Ferrara et al., 1991). The 16 kDa fragment is missing helix 4, which in GH and PL contains many of the receptor binding determinants. Therefore, the lack of this region in the 16 kDa PRL suggests that the manner in which this PRL variant binds to its own receptor may be distinct.

1.6.3.2 Site-Specific Mutants of PRL

Sequence comparisons of growth hormones, placental lactogens, and prolactins revealed several clusters of residues whose sequences were conserved in the lactogenic hormones but not in the non-lactogenic proteins of this group. The locations of these regions are the N-terminus of putative helix 1, the C-terminal part of the loop connecting putative helices 1 and 2, the middle of putative helix 2, and the N-terminal section of the loop connecting putative helices 3 and 4. The existence of these "lactogen-specific" residues suggested that lactogenic activities could be specifically associated with these portions of the hormones (Nicoll et al., 1986). However, as
mentioned previously (see section 1.6.1.1) this sequence comparison was unable to define the functional contribution of specific residues because the sequences of the hormones examined do not vary systematically. The role of individual amino acids is best determined through chemical modification and/or site-directed mutagenesis.

The contribution of arginine residues to binding of PRL to lactogen receptors was examined by treating oPRL with 1,2-cyclohexanedione which selectively modifies arginines: modification of the hormone resulted in decreased binding to receptors (Cymes et al., 1994). The reduction in affinity appeared to be due to modification of two arginine residues which reacted with the reagent faster than the other two arginines. The identity of the two more reactive arginines could not be precisely determined: they could be any of residues 21, 125, 176, and 177. Chemical treatment was also used to determine the importance of the three disulfide bonds of PRL to biological activity. Selective reduction and alkylation of the disulfide bonds of oPRL revealed that neither the N- nor C-terminal loop is required for mammotrophic activity. However, cleavage of all three disulfide bridges abolished biological activity, suggesting that the disulfide bridge joining the loop region between helices 1 and 4 with helix 4 was crucial for hormone activity (Doneen et al., 1979). The importance of this disulfide bond was confirmed when site-directed mutagenesis was used to replace the cysteine residues with serines in either bPRL (C58 and C174, Luck et al., 1992) or hPRL (C58, Goffin et al., 1992). The resulting mutant protein was unable to stimulate growth of Nb2 cells. Replacing the cysteines which form the N- and C-terminal loops with serines had no effect on the mitogenic activity of the hormone. The function of the C58 - C174 bond may be to maintain the proper spatial arrangement of the receptor binding determinants: disruption of the bridge could allow portions of the hormone which are normally held together to separate, and this could affect the ability of the hormone to bind to the receptor and induce mitogenesis.

The importance of the loop region connecting putative helices 1 and 2 to the bioactivity of hPRL was investigated by alanine-scanning mutagenesis (Goffin et al., 1992). Ten alanine mutants were generated in order to determine whether any of these residues is important for either binding of the hormone to the Nb2 lactogenic receptor or stimulation of Nb2 cell growth.
Binding affinities of the hPRL mutants was determined by measuring the ability of each mutant to compete with 125I-labeled unmodified hPRL for binding to Nb2 cell homogenates. Mutation of residues P66 and K69 had the most disruptive effect on the receptor binding and mitogenic ability of the hormone. The concentration at which half-maximal displacement of the 125I-labeled hPRL occurred (the IC50) was 3.3-fold higher for P66A and 10-fold higher for K69A than for unmodified hPRL. Similarly, the bioactivity of P66A, as measured by the ability of the mutants to stimulate propagation of the Nb2 cells, was 25% that of wild-type and the bioactivity of K69A was lower than that of wild-type hPRL by two orders of magnitude. Mutation of the other residues within the loop region did not severely affect hormone activity; in fact, S61A and Q74A were more active than wild-type hPRL (122% and 177%, respectively) and exhibited IC50 values slightly lower than that of unmodified hPRL. Thus, as for hGH in its interaction with both the hGHbp and the hPRLbp, the loop region connecting putative helices 1 and 2 of hPRL appears to be involved in receptor binding. However, the specific residues involved in binding to the receptor differ between these related hormones: amino acids I58, S62, and N63 of hGH (corresponding to L63, E67, and D68 of hPRL) are important for binding to the hPRLbp (Cunningham and Wells, 1991) while P66 and K69 of hPRL are required for binding (Goffin et al., 1992). Interestingly, while R64 of hGH, which is equivalent in position to K69 of hPRL, is not required for binding of hGH to the hPRLbp, it is involved in the interaction between hGH and the hGHbp (Cunningham and Wells, 1989).

The contribution of putative helix 4 to receptor binding and the bioactivity of hPRL was investigated using a novel approach. Instead of mutating specific hPRL residues, the activity of a hPRL molecule containing an additional nine residues at the C-terminus was examined (Goffin et al., 1993). The conformation of the mutant protein was assessed by circular dichroism (CD) and Fourier-transform infrared spectroscopy: the lack of any major differences in the results obtained from the mutant and from wild-type hPRL suggested that the additional nine residues did not affect the global folding of the hormone. However, the presence of the extra amino acids severely affected the ability of hPRL to bind to the Nb2 lactogenic receptor as indicated by a requirement for a 25- to 30-fold greater concentration of the mutant hPRL than of unmodified
hPRL for 50% displacement of $^{125}$I-labeled unmodified hPRL (the IC$_{50}$). In addition, the ability of the mutant to stimulate the growth of Nb2 cells was less than 3% that of wild-type hPRL. Although the authors did not precisely determine the three-dimensional location for this tail with respect to the native hormone, modeling studies suggested that the additional nine amino acids at the C-terminus lay within a small pocket defined by the N-terminal portion of putative helix 1, the C-terminal portion of putative helix 4, and the C-terminal half of the large loop between putative helices 1 and 2. This location is well away from K69, previously shown to be critical for binding of the hormone to the receptor, and the deleterious effect of the tail on hormone activity cannot therefore be attributed to disruption of this important interaction. The results suggest that the region of hPRL which comprises the small pocket described above might form part of the binding site of hPRL.

1.6.3.2.1 Site-Specific Mutants of bPRL

A structure-function analysis of bPRL was made possible by the development of a system for the efficient production of met-bPRL, that is, the mature 199 amino acid hormone with a methionine residue at the N-terminus, in transformed E. coli cells (Luck et al., 1986). The sequence encoding the signal peptide was removed from the 982-bp bPRL cDNA of clone pBPRL 72 (Sasavage et al., 1982) (Figure 6) as was most of the sequence 5' to the ATG initiation codon. The resulting cDNA, consisting of the entire coding sequence of mature bPRL plus six nucleotides 5' to the initiation codon, was cloned as a 686 bp insert into the EcoRI site of a modified pEMBL 8(+) vector [designated $\Delta^{13}$ pEMBL 8(+)]. This vector contained the consensus Shine-Delgarno sequence AGGA overlapping with a unique EcoRI recognition site (Luck et al., 1986). Very little met-bPRL was produced following transformation of E. coli with this plasmid. In an effort to determine the reason for the lack of met-bPRL production the bPRL coding sequence was fused to the N-terminus of the wild-type E. coli lacZ sequence. E. coli cells containing this plasmid synthesized large amounts of a lacZ-bPRL fusion protein, suggesting that the low level of production of met-bPRL from the $\Delta^{13}$ pEMBL 8(+) was most likely a consequence of the sequence 5' to the initiation codon of lacZ or of the sequence immediately downstream. Therefore, the sequence around the initiation codon for met-bPRL was replaced by
Figure 6. Nucleotide sequence of the mRNA coding for bPRL and its amino acid sequence. The mRNA sequence was deduced from pBPRL72 (Sassave et al., 1982) with changes in the third positions of codons 66, 76, and 137 as described (Luck et al., 1986). The coding portion of bovine preprolactin (690 bp) starts from the methionine AUG codon labeled -30 and terminates with the ochre UAA codon, labeled OC. The beginning of the processed bPRL protein is the threonine at position +1. The polyA sequence, not indicated, is at the extreme 3' end of the sequence.
one containing nucleotides from the consensus sequence around the initiation codon of *E. coli* genes. Transformation of *E. coli* cells with the resulting plasmid, pESP4, resulted in production of met-bPRL to approximately 5% of total cell protein.

Although the *E. coli* expression system described above resulted in efficient production of met-bPRL, the protein was produced in the form of insoluble inclusion bodies and this led to difficulties in isolating soluble, active protein. Therefore, as part of the work for this thesis, an attempt was made to avoid the problems associated with inclusion bodies by developing alternative expression systems for secreted bPRL, in yeast and in *E. coli*. Unfortunately, these did not result in the production of soluble active bPRL. However, several changes were made to the *E. coli* intracellular expression system that improved the production of met-bPRL.

Initial studies by Luck et al. (1989) to determine which bPRL residues interact with the lactogenic receptor, using the above *E. coli* expression system and site-directed mutagenesis, focussed on amino acids which are conserved in lactogens but are not found in the related but non-lactogenic bGH (Luck et al., 1989). Seven bPRL residues were identified by this comparison: four of these residues are within regions of the amino acid sequence previously identified as lactogen-specific by sequence comparisons (Nicoll et al., 1986). The seven residues were mutated to the corresponding bGH residues and the ability of the recombinant proteins to stimulate the lactogen-dependent growth of the Nb2 cells was determined. None of the single substitution mutations had a significant effect on the bioactivity of bPRL. However, a double mutant, S62T/T65A, had a bioactivity of 45% that of wild-type bPRL and the deletion of Y28 (located in the putative helix 1) abolished the bioactivity. The importance of the putative helical regions to the interaction of bPRL with the lactogenic receptor was investigated further in a study wherein single amino acids were specifically deleted from various regions of bPRL (Luck et al., 1990). Whereas removal of residues from non-helical regions had little effect on the bioactivity, removal of a residue from the centre part of a helical region led to essentially complete loss of bioactivity. It was suggested that this loss of bioactivity reflected an inability of the proteins to assume a native conformation (Luck et al., 1990).
1.7 Objective of Thesis Project

The members of the cytokine-hormone superfamily are involved in regulating many important biological processes, including growth, lactation, and the immune response. As such, there is a great deal of scientific, medical, and commercial interest in these proteins. For example, an understanding of the structure-function relationships of each protein with its own receptor or other receptors may potentially lead to improved methods of treatment of disorders such as acromegaly and hyperprolactinemia and to the design and production of variant hormones with altered binding specificity and improved pharmaceutical properties. Many studies to date have focussed on hGH, and because of the high homology among members of the growth hormone/prolactin/placental lactogen family, many of the results with hGH can be extrapolated to the other hormones. By extension, then, similar in-depth studies of other hormones will not only shed light on the particular hormones but also give more insights into the other members of the hormone family.

The homology between members of the growth hormone/prolactin/placental lactogen family extends beyond sequence and structure to the mechanisms by which each interacts with receptor proteins. The interaction of growth hormone, particularly hGH, with lactogenic and somatogenic receptors has been well studied, and the determination of the crystal structures of hGH complexed to the hGHbp and hPRLbp has provided a great deal of insight into how this protein elicits its effects. However, while the general mechanism of hormone-receptor binding appears to be conserved throughout this family, there are many differences with regard to the residues which form the functional binding sites. Ideally, an analysis of the crystal structures of the hormones and their receptors is required in order to obtain a full understanding of the interactions between the proteins; however, this information is not yet available for most members of the hematopoietic hormone family. In the absence of such structural information, a great deal of knowledge as to which residues are involved in eliciting biological responses from the receptors can be obtained from molecular biology techniques such as site-directed mutagenesis. In this thesis, site-directed mutagenesis coupled with \textit{in vivo} analysis using Nb2
cell cultures to measure mitogenic activity are used to investigate the structure-function relationships of bovine prolactin.

The studies which have been carried out on the interactions of bPRL with lactogenic receptors have confirmed that the mechanism of hormone-receptor interaction appears to be similar to that of growth hormone, but a complete definition of the prolactin residues which mediate its mitogenic and bioactivity is lacking. In this study of bPRL the residues involved in the mitogenic activity of this hormone in the Nb2 cell assay have been investigated in regions of the hormone that are homologous with functionally important regions of related proteins. The information obtained from the studies on growth hormone-receptor binding has identified the N-terminus of helix 1, the loop region between helices 1 and 2, a portion of helix 3, and the C-terminus of helix 4 as being involved in receptor binding. The focus of the current investigation was on bPRL residues within the loop joining putative helices 1 and 2 and within putative helices 3 and 4. Amino acids in these areas were selectively mutated and the ability of the variant proteins to stimulate the growth of Nb2 cell cultures was determined.

The results obtained in this study highlight both the similarities and the differences between the members of this hormone family. Although, as expected, the general areas of bPRL which appear to interact with the lactogenic receptor were found to be homologous to those involved in receptor binding in related proteins, there were differences with regard to specific residues. While this result was expected for more distantly related proteins such as bPRL and hGH it was unanticipated for the closely related hormones bPRL and hPRL. Clearly, although the mechanisms for binding and activation of the receptors in this hormone family are very similar, the exact details are different in many important ways. Thus, detailed structure-function analyses appear to be required for each member of the cytokine hematopoietin family in order to elaborate the precise details of hormone-receptor interactions.
MATERIALS AND METHODS

2.1 Materials

Restriction enzymes and DNA-modification enzymes were purchased from Boehringer Mannheim, BRL, New England Biolabs, Pharmacia, Promega, or USB. DNA sequencing kits (Sequenase™ Version 2.0) were obtained from USB and deoxynucleotides from Pharmacia. Rabbit anti-ovine PRL was obtained from USB and the horseradish peroxidase and alkaline phosphatase complexes of goat anti-rabbit IgG were purchased from Bio-Rad. The ECL™ kit was obtained from Amersham. Polyvinylidene difluoride (PVDF) microporous membrane (Immobilon-P) was obtained from Millipore. Film used for detection of chemiluminescence was purchased from Amersham (Hyperfilm-EC) and Du Pont (Reflection™) while film used for autoradiography was obtained from Kodak (X-Omat™) and Agfa (Curix RP-1). Authentic bPRL (USDA-bPRL-B-1) was a generous gift from Dr. D.J. Bolt of the USDA Animal Hormone Program. Fischer’s medium was purchased from Gibco and non-lactogenic horse (gelding) serum was from the National Biological Laboratory Ltd. All other reagents were of analytical grade and/or for biochemical use.

2.2 Strains and Growth Conditions

2.2.1 Bacterial Strains

The Escherichia coli strains used in this study are listed in Table 2.

2.2.2 Yeast Strains

The Saccharomyces cerevisiae strains used for production of bPRL were SR1069B (MATα his4-912 leu2-3 ura3-52) and XV700-24C (MATα leu2 ura3 pep4). Total RNA from strain S25 (MATα his4-912 ura3-52 leu2-3 leu 2-112) was used as a control for primer extension analysis.

2.2.3 Media for Growth of Bacterial and Yeast Strains

The liquid media used for growth of bacterial strains included YT (0.8% tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented with ampicillin (amp) at 100 µg/mL for selection of ampicillin resistant transformants; superbroth (3.2% tryptone, 2% yeast extract, 0.5% NaCl,
0.5% (v/v) 1 M NaOH) supplemented with 200 µg amp/mL; terrific broth (1.2% tryptone, 2.4% yeast extract, 0.4% (v/v) glycerol, 17 mM KH$_2$PO$_4$, 72 mM K$_2$HPO$_4$) supplemented with 200 µg amp/mL; and M9+B1 (50 mM Na$_2$HPO$_4$, 25 mM KH$_2$PO$_4$, 8.5 mM NaCl, 20 mM NH$_4$Cl, 10 mM glucose, 0.1 mM CaCl$_2$, 1 mM MgSO$_4$, 0.0001% thiamine) supplemented with 100 µg amp/mL. Transformants were selected by plating on YT + amp plates (made with 1.5% agar and containing 100 µg amp/mL).

Yeast strains were grown in complete YPD medium (1% yeast extract, 2% peptone, 2% dextrose) and in SD minimal media (0.67% yeast nitrogen base [without amino acids], 2% dextrose) supplemented with amino acids as required.

**Table 2. List of E. coli Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSH50</td>
<td>Δlac-pro ara thi F[traD36 proA$^+$ lacI$^q$ lacZΔM15]</td>
<td>Kunkel, 1985</td>
</tr>
<tr>
<td>HB2151</td>
<td>K12 ara Δlac-pro thi F[proA$^+$ lacI$^q$ lacZΔM15]</td>
<td>Carter et al., 1985</td>
</tr>
<tr>
<td>HB2154</td>
<td>K12 ara Δlac-pro thi mutL::Tn10 F[proA$^+$ lacI$^q$ lacZΔM15]</td>
<td>Carter et al., 1985</td>
</tr>
<tr>
<td>JM101</td>
<td>supE thi Δ(lac-proAB) F[traD36 proA$^+$ lacI$^q$ lacZΔM15]</td>
<td>Messing, 1983</td>
</tr>
<tr>
<td>JM105</td>
<td>supE endA sbcB15 hsdR4 rpsL thi Δ(lac-pro AB) F[traD36 proA$^+$ lacI$^q$ lacZΔM15]</td>
<td>Yanisch-Perron et al., 1985</td>
</tr>
<tr>
<td>Le392F</td>
<td>supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1 F[traD36 proA$^+$ lacI$^q$ lacZΔM15]</td>
<td>Borck et al., 1976</td>
</tr>
<tr>
<td>MV1193</td>
<td>Δ(lac-pro AB) rpsL thi endA specB15 hsdR4 Δ(srl-recA)306::Tn10(ief') F[traD36 proA$^+$ lacI$^q$ lacZΔM15]</td>
<td>Zoller and Smith, 1987</td>
</tr>
<tr>
<td>NM522</td>
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<td>Gough and Murray, 1983</td>
</tr>
<tr>
<td>RV308</td>
<td>su· ΔlacX74 gal IS11::OP308 rpsL</td>
<td>Schoner et al., 1985</td>
</tr>
<tr>
<td>RZ1032</td>
<td>HFrKL16 PO/45 [lysA(61-62)] Zbd-279::Tn10 lysA thi1 relA1 spoT1 supE44 dut1 ung1</td>
<td>Kunkel, 1985</td>
</tr>
<tr>
<td>TG1</td>
<td>supE hsdΔ5 thi Δ(lac-proAB) F[traD36 proA$^+$ lacI$^q$ lacZΔM15]</td>
<td>Gibson, 1984</td>
</tr>
<tr>
<td>TOPP1,2</td>
<td>rif$^r$ [F' proA$^+$ lacI$^q$ lacZΔM15 Tn10(ief')]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>TOPP3</td>
<td>rif$^r$ [F' proA$^+$ lacI$^q$ lacZΔM15 Tn10(ief')] kan$^r$</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>
2.3 Plasmids

The *E. coli* expression plasmid pESP4 (Figure 7) used for production of met-bPRL is a 4.7 kbp $\Delta^{13}$ pEMBL 8(+) vector containing a modified bPRL cDNA under the inducible control of the *E. coli* lac promoter (Luck *et al.*, 1986). As discussed in the Introduction, the cDNA was modified by removal of both a 5' nontranslated sequence and the signal sequence of preprolactin. In addition, the sequence around the ATG initiation codon was replaced with one that conformed as closely as possible to the consensus sequence in this region of *E. coli* genes (Luck *et al.*, 1986). As well as the gene for bPRL, the plasmid pESP4 contains the *E. coli* ori (for replication in *E. coli*), a marker for ampicillin resistance (for selection in *E. coli*), and the f1 intergenic region (for *in vivo* production of ssDNA by the filamentous bacteriophage R408). All bPRL variants used in this study were derived from pESP4 by site-directed mutagenesis.

Other plasmids used in attempts to obtain secreted bPRL from either *E. coli* or *S. cerevisiae* are described below in sections 2.12 and 2.13.

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**Figure 7.** Map of pESP4. The 4700 bp vector containing the bPRL coding sequence under control of the lac promoter is derived from pEMBL 8(+) as discussed in the Introduction.
2.4 Transformation of Cells

For high efficiency yeast transformation, spheroplasts of *S. cerevisiae* were transformed as described (Ausubel *et al.*, 1994).

Bacterial strains were transformed by the calcium chloride procedure or by electroporation. All CaCl$_2$-competent cells, except TOPP2 cells, were prepared and transformed essentially as previously described (Ausubel *et al.*, 1994). Cells from a 50 mL culture of mid-log phase *E. coli* cells were harvested by centrifugation at 2,000 x g for 8 min, gently resuspended in 25 mL ice-cold 50 mM CaCl$_2$, and incubated on ice for 30 min. Cells were again pelleted by centrifugation and gently resuspended in 3 mL ice-cold CaCl$_2$. For storage of competent cells 140 µL aliquots of the CaCl$_2$-competent cells were mixed with 60 µL ice-cold 50% glycerol and frozen at -70°C. For transformation, 10 - 50 ng plasmid DNA was added to 200 µL of the CaCl$_2$-competent cells, the cells were incubated on ice for 30 min and then heated at 42°C for 2 min. 6 - 8 µL of the suspension was plated directly on YT plates containing 100 µg amp/mL (YT + amp plates) for selection of transformants.

CaCl$_2$-competent TOPP2 cells, prepared as described above, were transformed as recommended by Stratagene (protocol supplied with competent cells). β-mercaptoethanol was added to 100 µL of competent TOPP2 cells to a final concentration of 25 mM. Cells were incubated on ice for 10 min prior to the addition of 10 - 100 ng plasmid DNA. Following an additional 30 min incubation on ice cells were heated at 42°C for 45 sec, incubated on ice for 2 min, and then diluted with 0.9 mL SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 20 mM glucose) and incubated at 37°C for 1 h with shaking. Transformants were selected by plating on YT + amp plates and incubating overnight at 37°C.

Electroporation was performed as recommended by Bio-Rad (protocol outlined in the Pulse Controller Instruction Manual). *E. coli* cells at early to mid-log (OD$_{600}$ 0.5 - 0.8) were harvested by centrifugation at 4,000 x g for 15 min, resuspended in 1 vol ice-cold dH$_2$O, centrifuged, resuspended in 0.5 vol ice-cold dH$_2$O, centrifuged, and then resuspended in 0.02 vol ice-cold 10% glycerol and centrifuged. The pellet was resuspended to 0.002 - 0.003 vol in 10% glycerol.
For transformation, 1 - 2 μL of plasmid DNA in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added to the electro-competent cells, the mixture incubated on ice for 1 min and then pulsed at 2.5 kV, 25 μF, and 200 Ω using 0.2 cm cuvettes in a Gene Pulser® Transfection Apparatus (Bio-Rad). A 1 mL volume of SOC medium was added to the mixture and aliquots were plated on YT + amp plates for selection of transformants.

2.5 Isolation of Plasmid DNA

Plasmid DNA was isolated from bacteria by a modified version of the alkaline lysis procedure (Ausubel et al., 1994; Pelham, 1985). For small scale plasmid production 1.5 mL cultures were grown overnight in YT + amp, the cells harvested by centrifugation in a microcentrifuge at maximum speed (12,700 x g), and the cell pellets resuspended in 100 μL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After incubation at room temperature for 5 min, 200 μL of 1% SDS, 0.2 M NaOH were added and the solution was incubated on ice for 5 min. Then 150 μL of 5 M potassium acetate, pH 4.8 was added and the mixture incubated on ice for an additional 5 min. The DNA in the supernatant was precipitated by the addition of 900 μL of 95% ethanol, collected by centrifugation for 2 min in a microcentrifuge, then dissolved in 100 μL TE plus 100 μL 5 M LiCl and incubated on ice for 5 min. Following centrifugation for 5 min in a microcentrifuge the supernatant was decanted and the DNA precipitated with 400 μL of 95% ethanol. The DNA was collected by centrifugation in a microcentrifuge for 2 min, washed with 1 mL of 70% ethanol, centrifuged, and dried. The pellets were dissolved in 50 μL TE.

Larger amounts of plasmid DNA (from 50 mL cultures) were prepared by a slightly modified version of another procedure (Sambrook et al., 1989). Cells were harvested by centrifugation (12,000 x g for 10 min), the pellets resuspended in 20 mL ice-cold 50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0 and centrifuged (12,000 x g for 10 min). The cells then were resuspended in 3 mL 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0 containing 5 mg lysozyme/mL and incubated at room temperature for 10 min. To this was added 6 mL of 1% SDS, 0.2 M NaOH and the suspension was incubated on ice for 5 min. Then 4.5 mL of 5 M potassium acetate, pH 4.8 was added and the mixture incubated for an additional 5 min on ice.
The suspension was centrifuged (17,500 x g for 30 min) and the DNA precipitated by adding 8 mL isopropanol to the supernatant and incubating for 30 min at room temperature. The DNA was pelleted by centrifugation (8,000 x g for 30 min) in a warm rotor and resuspended in 1,200 μL TE plus 1200 μL 5 M LiCl. The suspensions were incubated on ice for 5 min and then centrifuged in a microcentrifuge for 5 min. The DNA was precipitated by the addition of 2 vol 95% ethanol and collected by centrifugation for 2 min in a microcentrifuge. The DNA pellet was dissolved in 162 μL TE, cleaned by the addition of 18 μL RNase A (20 μg/mL, 30 min at 37°C) followed by 20 μL proteinase K (50 μg/mL, 30 min at 37°C), extracted once with phenol/CHCl₃ (1:1), and precipitated by the addition of 0.5 vol 7.5 M ammonium acetate and 2 vol 95% ethanol. The DNA was collected by centrifugation for 5 min in a microcentrifuge, washed with 1 mL of 70% ethanol, and dissolved in TE buffer.

2.6 Isolation of Single-Stranded DNA

Single-stranded DNA (ssDNA) templates from plasmids containing the f1 ori were prepared for DNA sequencing (from strain HB2151 containing the F factor genetic element (F') which allows the cells to be infected by filamentous phages such as R408) and site-directed mutagenesis (from the dut ung F' strain RZ1032 for uracil-containing ssDNA) as described (Dente et al., 1983). Briefly, YT + amp medium was inoculated with cells from a single colony of freshly transformed E. coli and the culture was incubated at 37°C with shaking for approximately 1 h (OD₆₀₀=0.2) prior to the addition of R408 helper phage at a multiplicity of infection of 20:1. Following infection, the R408 phage packages the f1 ori-containing plasmid DNA and produces ssDNA. The superinfected cells were incubated at 37°C with shaking for an additional 5 - 6 h. Phage particles were precipitated from the supernatant with 0.25 vol 20% PEG 8,000, 2.5 M NaCl at room temperature for 15 min and collected by centrifugation in a microcentrifuge (12,700 x g for 5 min). The phage pellets were resuspended in TE, extracted with phenol/CHCl₃ (1:1), and the ssDNA precipitated by the addition of 0.1 vol 3 M sodium acetate and 2 vol 95% ethanol. The ssDNA was collected by centrifugation for 5 min in a microcentrifuge, washed with 1 mL 70% ethanol, dried, and dissolved in TE buffer.
2.7 Site-Directed Mutagenesis

Site-directed mutagenesis (Zoller and Smith, 1982) was performed essentially as described (Kunkel et al., 1987). Phosphorylated oligonucleotides synthesized using an Applied Biosystems 380B or 380A DNA synthesizer were purified either on C18 Sep Pak cartridges (Millipore) (Atkinson and Smith, 1984) or by extraction with 30% NH4OH and butanol (Sawadogo and Van Dyke, 1991). The mutations created by oligonucleotide-directed mutagenesis and the oligonucleotides used are listed in Table 3.

The procedure used for site-directed mutagenesis is as follows. One pmol of the oligonucleotide was mixed with 0.1 - 0.5 pmol of the uracil-containing ssDNA template (U-ssDNA, prepared from the dut ung E. coli strain RZ1032) in 10 μL of core buffer (500 mM NaCl, 100 mM Tris-HCl, 100 mM MgCl2, 10 mM DTT, pH 8.0). Annealing was promoted by heating for 5 min at 55°C followed by a 10 min incubation at room temperature. The extension/ligation step was catalyzed by 1 U DNA polymerase I (Klenow fragment) and 1 U T4 DNA ligase in ligase buffer (660 mM Tris-HCl, 50 mM MgCl2, 50 mM DTT, pH 7.5) with added ATP (to 0.5 mM) and dNTPs (to 0.5 mM). The final reaction volume was 20 μL. The reaction was incubated for 2 h at room temperature. CaCl2-competent E. coli HB2151 cells were transformed with 5 μL aliquots of the mutagenesis mixture while 0.5 μL aliquots were used to transform electro-competent HB2151 cells.

2.8 DNA Sequencing

DNA sequence analysis was performed on ssDNA templates (isolated from the E. coli strain HB2151 as described above) using the Sanger dideoxy chain termination method (Sanger et al., 1977) with modified T7 DNA polymerase (Sequenase®) and [α-35S]thio-dATP according to the procedure recommended and supplied by the manufacturer (USB). dITP nucleotides were used to avoid compression regions within the bPRL gene. The sequence was visualized by autoradiographic detection on Curix RP-1 film (Agfa).
<table>
<thead>
<tr>
<th>Oligo</th>
<th>Mutagenic Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mutation(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH4</td>
<td>CCATTTGGACAAACTGGTGTGCGAGATATTTGGCTGCA AAACCCAGCAAAAGGAAGAAAGCTTGCAAAAGC ATAATAGAATCCTGTTG</td>
<td>Attach invertase signal sequence to 5' end of bPRL gene</td>
</tr>
<tr>
<td>MH6</td>
<td>CAGGTCCATTTGGACCAACTGGTGTGGCCGCTA CGGTGCGCAAAACCA</td>
<td>Attach bPRL gene in-frame to ompA signal sequence</td>
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<sup>a</sup> The sequences of the mutagenic primers are presented from the 5' to the 3' end. Each primer was phosphorylated at the 5' end during synthesis.

<sup>b</sup> Mixed oligonucleotides are shown with the two possible bases at each mixed position divided by a "/".
2.9 Preparation and Analysis of RNA

2.9.1 Isolation of Yeast RNA

Where appropriate, solutions were treated with diethylpyrocarbonate (DEPC) as follows. To inhibit RNase activity, 0.2 mL of DEPC was added per 100 mL of the solution to be treated and the mixture shaken vigorously to get the DEPC into solution, stirred for approximately 1 h at room temperature, and then autoclaved. In order to collect yeast cells for extraction of RNA, 50 mL of *S. cerevisiae* culture grown in SD selective medium to an OD$_{600}$ of 0.5 - 1 were filtered through a 0.45 µm nitrocellulose filter. The harvested cells were then washed with 500 µL of breakage buffer (0.2 M Tris-HCl, 0.5 M NaCl, 10 mM EDTA, pH 7.5) and resuspended in 200 µL of the same buffer. Acid-washed glass beads and 200 µL of 4:4:1:1 phenol/CHCl$_3$/10% SDS/3 M potassium acetate, pH 5.5 were added and the suspension was vortexed vigorously for 5 min. The organic phase was removed and discarded and the aqueous phase was extracted twice more as described above. The aqueous phase was then extracted once with CHCl$_3$. The RNA in the aqueous phase was precipitated with 0.4 vol 5 M ammonium acetate, pH 7.4 and 2.5 vol 95% ethanol, washed with 70% ethanol, dried, and dissolved in 40 µL dH$_2$O by heating at 85°C for 2 min. The amount of RNA was estimated by the A$_{260}$ (using the formula $C$ (µg/mL) = A$_{260}$/0.025) and adjusted to 10 µg/mL for use in primer extension analysis.

2.9.2 Isolation of *E. coli* RNA

Total *E. coli* RNA was isolated according to published methods (Ausubel et al., 1994). Where appropriate, solutions were treated with DEPC as described above and autoclaved in order to inhibit RNase activity. 100 mL cultures of TOPP2 transformed with vectors expressing various bPRL mutants were grown to log phase in terrific broth containing 200 µg amp/mL and 3 mM IPTG. Cell growth was stopped by the addition of 5 mL of stop buffer (200 mM Tris-HCl, 20 mM EDTA, 20 mM sodium azide, pH 8.0) and the cells were harvested by centrifugation at 5,500 x g for 5 min. The pelleted cells were resuspended in 2 mL lysing solution (8% w/v sucrose, 5% v/v Triton X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 7.0) plus 100 µL 200 mM vanadyl ribonucleoside complex (VRC), extracted with 1 mL phenol, and then extracted with 1 mL CHCl$_3$. Nucleic acids were precipitated from the aqueous layer with 0.1 vol 3 M sodium
acetate and 2 vol ice-cold 100% ethanol, collected by centrifugation at 10,000 x g for 10 min, and resuspended in 2 mL 10 mM VRC. The nucleic acid in the VRC solution was extracted twice more with 1:1 phenol/CHCl$_3$ and precipitated with sodium acetate and ethanol as described above. The nucleic acid pellet was then resuspended in 2 mL DEPC-treated dH$_2$O, 1 g CsCl was added, and 2.25 mL of the solution was layered onto a 0.75 mL CsCl cushion (5.7 M CsCl in 100 mM EDTA, pH 7.0) in a 13 x 51 mm TLA-100.3 polycarbonate tube. The RNA was pelleted by centrifugation for 1 h at 80,000 rpm (280,000 x g) at 20°C in a TLA-100.3 rotor. The RNA was resuspended in 0.36 mL DEPC-treated dH$_2$O, 36 µL 3 M sodium acetate and 2.5 vol ice-cold 100% were added, and the RNA was precipitated by a 20 min incubation at -70°C followed by centrifugation in a microcentrifuge for 5 min at 4°C. The pellet was washed with 1 mL of ice-cold 70% ethanol, centrifuged in a microcentrifuge for 5 min at 4°C, dried, dissolved in 200 µL DEPC-treated dH$_2$O and quantified by the A$_{260}$. The concentration was adjusted to 4 µg/mL in DEPC-treated dH$_2$O and the RNA solution stored at -70°C.

2.9.3 Primer Extension Analysis of Yeast RNA

One pmol of oligonucleotide primer was incubated in 20 µL PNK buffer (100 mM Tris-HCl, 10 mM MgCl$_2$, 10 mM DTT, pH 8.0) containing 9 U T4 polynucleotide kinase and 3 pmol γ-$^{32}$P ATP (10 µCi/µL) for 30 min at 37°C. The reaction was stopped by incubation at 65°C for 5 min. The labelled primer was annealed to the RNA by incubating 1 µL of the kinase-labelled primer with 5 µL of yeast RNA (10 µg/µL) and 1 µL buffer A (500 mM Tris-HCl, 1 M KCl, 100 mM MgCl$_2$, pH 8.5) at 85°C for 2 min. The reaction was then incubated on ice for 10 min. The extension reaction was carried out by adding 1 µL AMV reverse transcriptase (diluted to 1 U/µL in 50 mM Tris-HCl, 100 mM KCl, 10 mM DTT, 10 mM MgCl$_2$, pH 8.5) and 3 µL of 500 µM dNTPs, 10 mM DTT and incubating at 42°C for 1 h. The reaction was stopped by the addition of 10 µL formamide dye mix (80% formamide, 10 mM EDTA pH 8.0, 1 mg xylene cyanol FF/mL, 1 mg bromophenol blue/mL) and 1 µL 0.2 M NaOH. The final mixture was boiled for 5 min and analyzed by denaturing polyacrylamide gel electrophoresis. The gel was soaked in a solution of 10% glacial acetic acid, 12% methanol, dried, and exposed to film for visualization of the bands.
2.9.4 Competitive PCR Analysis of E. coli RNA

Detection and quantitation of mRNA species from selected recombinant bPRL variants was performed by the sequential use of reverse transcriptase and the polymerase chain reaction (PCR) (Foley et al., 1993; Gilliland et al., 1990; Perrin and Gilliland, 1990). Briefly, RNA isolated from E. coli cells expressing recombinant bPRL mutants was reverse transcribed with AMV reverse transcriptase. The resulting cDNA was then amplified by PCR in a reaction mixture containing a known amount of DNA from a bPRL variant containing the signal sequence for the yeast invertase gene (pMH4).

For the preparation of the cDNA, 100 ng RNA (from a 4 µg RNA/mL solution prepared as described above) was mixed with 20 pmol of bPRL primer, heated at 65°C for 10 min, and then reverse transcribed in a reaction mixture containing 20 U RNasin® ribonuclease A inhibitor (Promega), 25 U AMV reverse transcriptase, and 10 nmol each dNTP in 20 µL total volume of reverse transcriptase buffer (50 mM Tris-HCl, 8 mM MgCl₂, 30 mM KCl, 1 mM DTT, pH 8.5) for 1 hour at 42°C. The reaction mixture was added to 200 µL of a PCR master mixture containing 3 mM MgCl₂, 44 pmol each primer, 200 µM dNTPs, and 3.75 U Taq DNA polymerase in PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4). The master mix concentrations were adjusted to account for input primer and dNTPs from the reverse transcription reaction. 20 µL aliquots of the PCR mixture were added to 10 tubes each containing 5 µL of a dilution series of pMH4 plasmid DNA in PCR buffer. The 25 µL reaction volumes were overlayed with oil, heated at 94°C for 2 min, and 25 cycles of PCR were performed (94°C for 25 sec, 52°C for 30 sec, and 72°C for 1 min) followed by a 5 min extension at 72°C. An aliquot of each reaction mixture was subjected to electrophoresis in a 1.9% MetaPhor™ agarose gel (FMC BioProducts) prepared in TBE buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA). For all PCR reactions the controls included (i) reverse transcription reaction mixture from E. coli cells lacking the bPRL expression plasmid; (ii) no added cDNA; (iii) pMH4 and pESP4 plasmid DNA; (iv) cDNA not mixed with competitor pMH4; and (v) reverse transcription reaction mixture from which AMV reverse transcriptase was omitted.
2.10 Isolation of Met-bPRL

The total concentration of protein in extracts from various steps in the isolation procedure for bPRL was determined using the Lowry protein assay (Lowry et al., 1951).

2.10.1 Extraction of Met-bPRL and Met-bPRL Variants

Transformed E. coli cells (generally strains HB2151 or TOPP2) from which met-bPRL or met-bPRL variants were to be isolated were grown, lysed, and extracted essentially as previously reported (Luck et al., 1989) with minor modifications detailed below. Overnight cultures of transformed cells grown in 1.5 mL terrific broth + 200 μg amp/mL were diluted 1:100 into 100 mL of terrific broth containing 200 μg amp/mL and 3 mM IPTG. Cultures were incubated at 37°C with shaking (275 rpm) for 16 h and the cells were harvested by centrifugation (8,000 x g for 10 min). The cells were resuspended in 20 mL cold 0.1 M sodium phosphate buffer, pH 7.4, centrifuged (8,000 x g for 10 min), and resuspended in 20 mL 0.1 M sodium phosphate buffer, pH 7.4, containing 40 mM DTT and 1 mM phenylmethylsulfonylfluoride (PMSF). The cells in the suspension were lysed by a single passage through a French pressure cell at maximum pressure (20,000 psi). The insoluble material was collected by centrifugation at 20,000 x g for 30 min and resuspended using a Dounce homogenizer in 8 mL cold 0.1 M sodium phosphate buffer, pH 7.4, containing 5 mM DTT. The suspension was divided into 1 mL aliquots which could be stored indefinitely at -70°C. To remove some contaminating E. coli proteins 32 μL of 10% sodium desoxycholate were added to 800 μL of the suspension and the mixture was incubated at 37°C for 1 h. The suspension was centrifuged in a microcentrifuge at maximum speed for 10 min. The pellets were washed by the addition of 1 mL of dH2O followed by vigorous vortexing and the insoluble material was collected by centrifugation in a microcentrifuge for 10 min. Using a 22 gauge needle attached to a 1 mL syringe each pellet was resuspended in 500 μL 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% N-lauryl sarcosine (sarcosyl). Care was taken to avoid excessive aeration of the solutions. The suspensions were incubated overnight at 4°C in order to extract the met-bPRL. The insoluble material was then removed by 10 min centrifugation in a microcentrifuge and the supernatants, containing the solubilized met-bPRL, stored at 4°C.
2.10.2 Renaturation of Extracted Met-bPRL and Met-bPRL Variants

Renaturation of met-bPRL and its variants was carried out essentially as previously described (Luck and Huyer et al., 1992). The extracts of met-bPRL and its variants in 0.1% sarcosyl (475 μL volumes, prepared as described above) were diluted with 950 μL 0.1 M sodium borate, pH 10, containing 0.1% sarcosyl. In order to promote renaturation by air oxidation the samples were incubated at room temperature for 4 h in Eppendorf microcentrifuge tubes with the lids open. The samples were then neutralized by addition of 42 μL 6 N HCl. The renatured met-bPRL extracts were stored at 4°C prior to quantification by Western blot analysis. Samples could be stored in this state for approximately 2 weeks without loss of bioactivity. Following quantitation, samples for bioassay were diluted to 1 μg hormone/mL in Fischer's medium containing 10% gelding serum and 15 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]) buffer and stored at -20°C. Once diluted into the Fischer's medium the met-bPRL samples could be stored for long periods (e.g., 1 year) at -20°C; they retained bioactivity through repeated freeze-thaw cycles.

2.10.3 Quantification of Met-bPRL and Met-bPRL Variants

The concentration of renatured met-bPRL and its variants in the 0.1% sarcosyl extracts of E. coli cells (obtained as described above) was determined using Western blot analysis essentially as previously described (Luck and Huyer et al., 1992). Aliquots of the extracts and of standard pituitary bPRL (USDA-bPRL-B-1) (diluted in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% sarcosyl) were subjected to discontinuous SDS-PAGE (Laemmli, 1970) and analyzed by Western blotting. Samples were diluted in 2X sample buffer (0.2 M Tris-HCl, pH 6.8, 5% SDS, 30% glycerol) without β-mercaptoethanol (to provide non-reducing conditions) and loaded onto a discontinuous polyacrylamide gel (4% stacking gel over a 13% separating gel). Electrophoresis was carried out at room temperature using currents of 14 mA through the stacking gel and 28 mA through the separating gel. Following electrophoresis the separated proteins were transferred to a polyvinylidene difluoride (PVDF) microporous membrane at 100 mA for 2 - 2.5 h at 4°C using a Tris/glycine/methanol transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol). Blots were blocked in blotto (5% Carnation® instant skim milk powder

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in TBS-Tween [20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6]) for 1 h to overnight. The bPRL bands were visualized using enhanced chemiluminescence. The Western blot protocol followed was that recommended and provided for use with the Amersham ECL™ system. The primary antibody used was polyclonal rabbit anti-ovine PRL (USB) (diluted 1:10,000 in TBS-Tween), and the secondary antibody was the horseradish peroxidase conjugate of goat anti-rabbit IgG (Bio-Rad) (diluted 1:5,000 - 1:10,000 in TBS-Tween). Following exposure of the treated membrane to the detection solution the bands were visualized on Hyperfilm-EC (Amersham) or on Reflection™ autoradiography film (Du Pont). The densities of the bands of the non-reduced pituitary bPRL standards and of the corresponding bands of the met-bPRL variants were measured using a computerized laser scanning densitometer (Model 300A, Molecular Dynamics). Alternatively, the light emitting from the bPRL bands was detected using a high performance luminescence imaging system (Luminograph LB 980, EG&G Berthold). The amount of met-bPRL or met-bPRL variant in each extract was estimated by reference to a standard curve constructed from the densities of the bands, or the amount of light emitted, of the various concentrations of the pituitary bPRL standard.

Western blot analysis was also performed using a colourimetric system with alkaline phosphatase-linked secondary antibody (Harlow and Lane, 1988). Following electrophoresis the separated bands were transferred to nitrocellulose using the conditions described above. The nitrocellulose blot was blocked in incubation buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 5% skim milk powder, incubated with polyclonal rabbit anti-oPRL (diluted 1:2,000 with incubation buffer with 2.5% skim milk powder), and then with the alkaline phosphatase conjugate of goat anti-rabbit IgG (Bio-Rad) (diluted 1:1,500 with incubation buffer with 2.5% skim milk powder). The blot was washed with incubation buffer containing 0.25% sarcosyl. For visualization of the bands the blot was immersed in 30 mL 0.1 M NaHCO₃, 1 mM MgCl₂, pH 9.8, 9 mg nitro blue tetrazolium (NBT, made as a 50 mg/mL solution in 70% dimethylformamide) and 4.5 mg bromochloroindolyl phosphate (BCIP, made as a 25 mg/mL solution in dimethylformamide) was added, and the blot was incubated at room temperature until the bands were visible (approximately 10 min).
2.10.4  \textit{In Vitro} Bioassay of Met-bPRL and Met-bPRL Variants

Extracts of met-bPRL and its variants, diluted to 1 \( \mu \text{g/mL} \) in Fischer's medium containing 10\% gelding serum and 15 mM HEPES buffer, were assayed for mitogenic activity using the Nb2 lymphoma cell proliferation assay (Tanaka \textit{et al.}, 1980). The ability of the met-bPRL variants to stimulate the growth of lactogen-dependent Nb2 lymphoma cell cultures was compared with that of the pituitary bPRL standard preparation. The met-bPRL preparations were assayed in a series of dilutions to cover the growth response of the cells in a 48 h period to lactogens in the useful working range of 0.01 - 0.25 ng/mL. Cells were counted with a Coulter electronic cell counter. Mitogenic activities of the met-bPRL variants are expressed as percentages of the bioactivity of the pituitary bPRL standard.

2.11 Analysis of Production of Met-bPRL at Different Temperatures

\textit{E. coli} strains HB2151, Le392F', and TOPP2 transformed with pESP4 were incubated with shaking in 5 mL of YT, superbroth, and terrific broth medium containing 100 \( \mu \text{g amp/mL} \) (for YT) or 200 \( \mu \text{g amp/mL} \) (for superbroth and terrific broth) and 5 mM IPTG at room temperature (23°C), 30°C, and 37°C for 15 - 17.5 h. Cells were harvested by centrifugation (12,000 \( \times \) \( g \) for 10 min), the cell pellets were resuspended in 20 mM Tris-HCl buffer, pH 8.0 containing 20 mM EDTA and 2 mg lysozyme/mL, and the cells lysed by four successive rounds of freezing in liquid nitrogen followed by thawing in a room temperature waterbath. After lysis the DNA was sheared by sonication using the microtip probe for two 10 sec bursts, cooling on ice between each burst. The suspensions were centrifuged in a microcentrifuge for 10 min and the soluble and insoluble fractions loaded on polyacrylamide gels for analysis by coomassie blue-staining and Western blotting.

2.12 \textit{E. coli} Secretion System

2.12.1 Plasmids

The vectors pompPRL and pompPRLF are derived from the \textit{E. coli} secretion vector pINIII-OmpA3 (Ghrayeb \textit{et al.}, 1984). They were constructed as part of the system for secretion of
bPRL from *E. coli*. All DNA manipulations were carried out according to published protocols (Ausubel *et al.*, 1994).

**pompPRLF**: The phage f1 ori was subcloned into pINIII-OmpA3 in order that single-stranded vector DNA would be obtained following infection of *E. coli* with the filamentous helper phage R408. The bPRL gene was subcloned as an *EcoRI-BamHI* fragment into pINIII-OmpA3, creating the vector pINPR. The phage f1 intergenic region (f1 ori) was subcloned from the yeast/*E. coli* shuttle vector pVT100U (*Vernet et al.*, 1987) into pINPR as follows. The plasmid pVT100U was digested with *EcoRI*, the ends of the linearized vector filled in by DNA polymerase I (Klenow fragment) and the 1.2 kbp fragment containing the f1 ori isolated following digestion with *NdeI*. The f1 ori-containing fragment was subcloned into pINPR that had been digested with *AvaI*, blunt-ended with DNA polymerase I (Klenow fragment) and then digested with *NdeI*. The vector pompPRLF was created by looping out the DNA sequence between the 3' end of the *ompA* signal sequence and the 5' end of the bPRL gene.

**pompPRL**: The f1 ori was removed from pompPRLF as an *EcoRI-BgIII* fragment, the ends filled in with DNA polymerase I (Klenow fragment) and the blunt ends ligated together with T4 DNA ligase.

The vectors pJL3 and pSW1 were obtained from Drs. Oudega and Luirink (Vrije Universiteit, Amsterdam, The Netherlands). pJL3 codes for chloramphenicol resistance and contains the bacteriocin release protein (BRP) gene under the control of the *lpp-lac* promoter-operator system. pSW1 codes for tetracycline resistance and contains the BRP gene under the control of the mitomycin C inducible pCloDF13 promoter.

### 2.12.2 Secretion System

The BRP expression plasmids pJL3 and pSW1 were co-transformed with the vector pompPRLF into *E. coli* JM101. The double transformants of pJL3 and pompPRLF were selected on YT plates containing chloramphenicol (20 μg/mL) and ampicillin (100 μg/mL) and the double transformants of pSW1 and pompPRLF were selected on YT plates containing tetracycline (12 μg/mL) and ampicillin (100 μg/mL). The isolates were analyzed for bPRL release following induction of BRP and bPRL as follows. An overnight culture of cells containing pJL3 and
pompPRLF was diluted 1:50 with YT medium containing 100 μg amp/mL and 20 μg chloramphenicol/mL. After 1.5 h incubation with shaking at 37°C IPTG was added to 40 μM, MgSO₄ was added to 10 μM and the incubation was continued for 3 h. An overnight culture of cells containing pSW1 and pompPRLF was diluted 1:50 with YT medium containing 100 μg amp/mL and 12 μg tetracycline/mL. After 1.5 h incubation with shaking at 37°C IPTG was added to 100 μM, mitomycin C was added to 20 ng/mL and the incubation at 37°C was continued for 3 h. IPTG induces the expression of the bPRL gene in pompPRLF and the BRP gene in pJL3, and mitomycin C induces the expression of the BRP gene in pSW1. Samples analyzed by SDS-PAGE and Western blotting were diluted in sample buffer containing β-mercaptoethanol and boiled for 5 min prior to being loaded on a polyacrylamide gel.

2.12.3 Fractionation Procedures for Periplasmic Proteins

To determine whether bPRL expressed from the vectors pompPRL and pompPRLF was exported to the periplasm, E. coli strains JM101 and RV308 were transformed with each of these vectors and the resulting cells fractionated in order to obtain the periplasmic contents. The cell fractionation procedures were essentially as previously described (Koshland and Botstein, 1980). Strains JM101 and RV308 were transformed with the plasmids pompPRL and pompPRLF. Duplicate cultures in 20 mL YT + amp were incubated with shaking at 37°C for 1.5 h prior to induction of one of the duplicates with 2 mM IPTG. The RV308 cells were incubated for an additional 4 h (final OD₆₀₀=3.8) and the JM101 cells incubated for an additional 7 h (final OD₆₀₀=2.8). Aliquots (1.5 mL) of the cultures were centrifuged in a microcentrifuge for 5 min, the pellets resuspended in sample buffer and saved as samples of total cell protein. Additional 1.5 mL aliquots of the cultures were centrifuged in a microcentrifuge for 5 min and the pellets placed on ice prior to the following fractionation procedures.

**Lysis by Osmotic Shock:** Cells were resuspended in 150 μL of cold 20% sucrose, 10 mM Tris-HCl, pH 7.5 and incubated on ice for 30 min. Then 0.5 μL of 0.5 M EDTA, pH 8.0 was added, the suspension incubated on ice for an additional 10 min. A 50 μL aliquot was removed, diluted with 2X sample buffer, and saved as the periplasm control. The remainder of the suspension was centrifuged in a microcentrifuge at 4°C for 5 min. The pellet was rapidly and
vigorously resuspended in 100 μL of ice-cold dH₂O, incubated on ice for 10 min, and centrifuged in a microcentrifuge at 4°C for 5 min. The supernatant was diluted with 2X sample buffer and saved as the periplasmic contents fraction. The pellet was resuspended in 100 μL of dH₂O plus 100 μL 2X sample buffer and saved as the periplasmic membrane fraction.

Formation of Spheroplasts: Cells were resuspended in 75 μL of ice-cold 0.1 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose (pH 8.0) and incubated on ice for 30 min. Then 7.5 μL of 2 mg lysozyme/mL and 75 μL cold dH₂O was added and the suspension incubated on ice for an additional 25 min. A third of each sample was removed, diluted with 2X sample buffer, and saved as the spheroplast control. 3 μL of 1M MgCl₂ was added to the remainder and the suspension centrifuged for 5 min in a microcentrifuge. The supernatant was diluted with 2X sample buffer and saved as the periplasmic fraction while the pellet resuspended in 100 μL dH₂O plus 100 μL 2X sample buffer and saved as the membrane fraction.

All samples were analyzed by SDS-PAGE and Western blotting.

2.13 Yeast Secretion System

The yeast bPRL expression vector pVTUP was constructed by inserting the gene for bPRL, with the signal sequence from the yeast invertase gene fused to the 5' end, into the yeast/E. coli shuttle vector pVT101-U (Vernet et al., 1987). The vector contains the E. coli ori (for replication in E. coli), a marker for ampicillin resistance (for selection in E. coli), the fl intergenic region (for production of ssDNA by filamentous phage), the URA3 gene (for selection in yeast), the yeast 2μ ori (for replication in S. cerevisiae), and the bPRL gene under the control of the constitutive ADH1 promoter. The bPRL gene was isolated as a EcoRI-BamHI fragment in which the EcoRI ends were blunted with DNA polymerase I (Klenow fragment) and subcloned into pVT100-U digested with PvuII and BamHI.

pVTUP was transformed into the S. cerevisiae strains SR1069B and XV700-24C. Saturated cultures of pVTUP in SR1069B and XV700-24C were diluted 1:10 into 25 mL of YPD medium and incubated with shaking at 30°C for 24 h. Cells were harvested by centrifugation at 5,000 x g for 10 min. PMSF was added to the supernatants to a final concentration of 0.5 mM
and the supernatants were then concentrated using a Centriprep®-10 concentrator (Amicon) to a
final volume of approximately 1 mL. The cell pellets were resuspended in 10 mL dH2O,
centrifuged as above, resuspended in 4 mL 100 mM EDTA (pH 8), 0.5% β-mercaptoethanol, and
incubated at 37°C for 15 min. The suspensions were centrifuged (5,000 x g for 10 min) and the
cell pellets resuspended in 100 μL 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 M sorbitol, 2 mM
DTT. Zymolyase was added to 7.5 U/mL and the suspensions incubated at 30°C with gentle
shaking for 1.75 h. The spheroplasts were centrifuged in a microcentrifuge for 5 min,
resuspended in 75 μL 18% Ficoll, 0.5 mM PMSF and centrifuged in a microcentrifuge for 5 min.
The pellets were twice resuspended in 100 μL dH2O and collected by centrifugation in a
microcentrifuge and then resuspended in 100 μL dH2O and stored on ice.

The various cell extracts were diluted with 2X sample buffer containing β-mercaptoethanol,
boiled for 5 min and loaded on a polyacrylamide gel for analysis by SDS-PAGE and Western
blotting.

2.14 Molecular Modelling of bPRL

The bovine prolactin primary sequence was aligned to that of human growth hormone.
Coordinates of homologous regions were transferred from hGH to bPRL using Homology
version 2.35 (Biosym Technologies). Loop regions with no steric overlap and with good
geometry were spliced into the bPRL chain once all residues in regions of regular secondary
structure had been modelled. In three cases, helices spanning the bPRL residues 95 - 102, 111 -
138, and 162 - 193 had to be manufactured as perfect (φ = -57°) α-helices in the Biopolymer
modules and individually docked into the growing bPRL structure. This intervention was
necessary due to differences in the number of residues present within certain regions of the hGH
and bPRL sequences. Fourteen N-terminal residues did not have homologous counterparts in
hGH and were not predicted to form a regular secondary structure; hence, these residues (T1 -
S14) were deleted from the bPRL model. Once all coordinates had been assigned, amino and
carboxy termini for the polypeptide were created. Steric overlap was reduced using 100 steps of
conjugate gradient minimization in the Discover module of the Biosym software suite.
Further refinement of the bPRL model was achieved using X-PLOR version 3.1 (Brünger, 1992). Two disulfide pairs (residues 58, 174 and 191, 199) were patched. The model was then regularized using three cycles of unrestrained conjugate gradient minimization and 0.5 psec of molecular dynamics at 300°K followed by a final minimization. The target energy function included improper angle, Van der Waals, and bond-length components. Subsequent analysis of backbone (phi, psi, omega) torsion angles and side-chain rotomers using the PROCHECK suite of programs (Laskowski et al., 1993) indicated that the model had favourable geometry. In addition, no solvent accessible gaps in the structure were present when a molecular surface was constructed in the program GRASP (Nicholls et al., 1993). Figures of the bPRL molecular model were produced using the program SETOR (Evans, 1993).
RESULTS

3.1 Production of Recombinant bPRL

Although the expression system originally developed for production of met-bPRL in *E. coli* strain HB2151 (Luck *et al.*, 1986, 1989) resulted in efficient production of the hormone, accumulation of the met-bPRL in insoluble inclusion bodies led to difficulties in obtaining bioactive protein. Therefore, in an attempt to avoid the problems associated with obtaining active met-bPRL from inclusion bodies several avenues were explored to obtain the hormone in a soluble form, including reducing the growth temperature of the transformed cells and developing alternative expression systems, in *Saccharomyces cerevisiae* and *E. coli*, for production of bPRL.

It has been shown that while certain human proteins (e.g., interferon-α2, interferon-γ, and the interferon-induced murine protein Mx) are normally found in aggregates when produced in *E. coli* grown at 37°C, they were produced in soluble form when growth temperatures in the range of 23 - 30°C were used (Schein and Noteborn, 1988). Aggregate formation was not dependent upon the proteins being synthesized in the *E. coli* cells: interferon-α2 could be aggregated *in vitro* by incubating the protein at 37°C with *E. coli* cell lysate obtained from growth at either 30°C or 37°C. This result suggested that the production of inclusion bodies may be linked to protein turnover in the *E. coli* cells, a process which is affected by temperature.

In order to test whether growth temperature affects the solubility of bacterially-produced bPRL, *E. coli* strains HB2151, Le392F', and TOPP2 transformed with the bPRL expression plasmid pESP4 were incubated at room temperature (23°C), 30°C, and 37°C. The soluble and insoluble fractions of the cells obtained by freeze-thaw lysis were analyzed by SDS-PAGE and Western blotting. A small amount of soluble bPRL was produced in cells grown in superbroth and terrific broth at 23°C and 30°C; however, the amount of hormone present was barely detectable on a coomassie blue-stained gel and comprised much less than 1% of the total cell protein. The vast majority of the bPRL produced at all three growth temperatures was in the form of insoluble aggregates (data not shown).
3.1.1 Yeast Secretion System

An expression vector for the production of bPRL in yeast, pVTUP, was created by fusing the 5' end of the bPRL coding sequence to the signal sequence from the yeast invertase gene and subcloning the product into a yeast/E. coli expression vector, pVT101-U (Vernet et al., 1987). Expression of the bPRL gene was under the control of the constitutive ADH1 promoter and the presence of an ampicillin resistance gene and the ura3 gene allowed selection of the plasmid in both E. coli and yeast, respectively. pVTUP was transformed into S. cerevisiae strains SR1069B and XV700-24C. XV700-24C, carrying the pep4 marker, is a protease-deficient strain defective in processing precursors of vacuolar enzymes (Hemnings et al., 1981). Following 24 h growth in YPD medium, cells were harvested by centrifugation and the culture supernatants concentrated. The contents of the cells were isolated by lysing spheroplasts formed by treatment with Zymolyase. Samples consisting of the concentrated culture supernatants and the cell contents were loaded on a denaturing polyacrylamide gel and analyzed by Western blotting using a colourimetric detection system, polyclonal rabbit anti-oPRL, and an alkaline phosphatase conjugate of goat anti-rabbit IgG. No bPRL was detected in the concentrated supernatants or in the fractions from the spheroplasting procedure (data not shown).

A primer extension experiment was performed in order to determine if the lack of bPRL production in S. cerevisiae was due to an absence of bPRL mRNA. Total RNA was isolated from strains SR1069B and XV700-24C transformed with plasmid pVTUP which contained the bPRL cDNA. As a negative control, RNA was isolated from strain SR1069B transformed with the parent plasmid pVT101-U. All RNA preparations were examined using the primers MH7, which hybridized specifically to the bPRL cDNA; CYH26 for the yeast ura3 gene; and P20 for the yeast leu2 gene. Controls included total RNA isolated from strains SR1069B and XV700-24C not transformed with any plasmid as well as total RNA isolated from ura+ and ura- strains of S. cerevisiae. Results from the analysis of the above RNA samples indicated that bPRL mRNA was present in the strain transformed with the bPRL expression vector pVTUP (see Figure 8 for results from strain SR1069B). The absence of bPRL in the S. cerevisiae strains containing the bPRL expression vector cannot be due to a lack of mRNA and may be due to inefficient
translation of the bPRL mRNA or to degradation of the hormone. These possibilities were not investigated and no further effort was made to obtain secreted bPRL from *S. cerevisiae*.

**Figure 8.** Primer extension analysis of bPRL mRNA in the *S. cerevisiae* expression system. Total RNA samples analyzed were: 1, from strain SR1069B not transformed with any plasmid; 2, from strain SR1069B transformed with the parent vector pVT101-U; and 3, from strain SR1069B transformed with bPRL expression vector pVTUP. Primers used were a, MH7, which hybridizes to the bPRL gene; b, CYH26, which hybridizes to the *ura3* gene; and c, P20, which hybridizes to the *leu2* gene. Lane 4 contains total yeast RNA from strain S25 carrying a promoter-less *ura3* gene (ura-) strain and lane 5 contains total yeast RNA from strain S25 carrying the *ura3* gene fused to the *cycI* promoter (ura+). Lanes 4 and 5 were probed with primer CYH26.

### 3.1.2 *E. coli* Secretion System

Secretion of bPRL into the *E. coli* culture medium was attempted using a system developed for the secretion of hGH (Hsuing *et al.*, 1989). The expression vector pompPRLF, containing an ampicillin marker and the bPRL gene fused to the *ompA* signal sequence under the control of the *lpp-lac* promoter-operator system, was constructed and co-transformed into *E. coli* HB2151 with the bacteriocin release protein (BRP) expression vectors pJL3 or pSW1. Dual antibiotic resistance (ampicillin plus chloramphenicol or tetracycline) was used to select for transformants carrying both the bPRL secretion vector and one of the BRP expression vectors. pJL3 codes for chloramphenicol resistance and contains the BRP gene under control of the *lpp-lac* promoter-
operator. pSW1 codes for tetracycline resistance and contains the BRP gene under control of the pCloDF13 promoter. Bovine PRL was detected in cells of HB2151 transformed either with pompPRLF alone or with pompPRLF plus pJL3 or pSW1 (Figures 9 and 10, section 1). However, no hormone was detected by Western blot analysis of concentrated samples of the culture medium (data not shown). In addition, bPRL was not present in the soluble fraction obtained following lysis of the cell cultures with a French pressure cell (Figures 9 and 10, section 2). Therefore, contrary to expectations, the bPRL joined to the ompA signal sequence was not present in a soluble form and did not appear to be exported to the periplasm. The bPRL was instead found associated with the insoluble material (Figures 9 and 10, section 3), suggesting that the hormone accumulated in inclusion bodies. The presence of two closely spaced bands at the position corresponding to bPRL that were reactive with the anti-PRL antibodies (Figure 10) may indicate cleavage of the signal sequence from part of the population of bPRL.

Figure 9. Coomassie blue-stained denaturing polyacrylamide gels of bPRL in various fractions from the E. coli secretion system. E. coli strain HB2151 was transformed with the bPRL expression vector, pompPRLF, alone or with pompPRLF plus one of the BRP expression vectors. Cultures were grown to stationary phase in YT + amp +/− 12 μg tetracycline/mL (for pSW1) +/− 20 μg chloramphenicol/mL (for pJL3). Samples analyzed were (1) total cell protein; (2) soluble portion following cell lysis by a French pressure cell; and (3) pelleted material following cell lysis. The expression vectors were (a) pompPRLF; (b) pJL3 and pompPRLF; and (c) pSW1 and pompPRLF. The bPRL standard is 1000 ng of pituitary bPRL. The upper band in the lane containing the standard bPRL corresponds to BSA, added as a stabilizer for the bPRL.
Figure 10. Western blot analysis of bPRL in fractions from the *E. coli* secretion system. *E. coli* strain HB2151 was transformed with the bPRL expression vector, pompPRLF, alone or with pompPRLF plus one of the BRP expression vectors. Cultures were grown to stationary phase in YT + amp +/- 12 μg tetracycline/mL (for pSW1) +/- 20 μg chloramphenicol/mL (for pJL3). Samples were loaded on a denaturing polyacrylamide gel, and analyzed by a colourimetric Western blot system using polyclonal rabbit anti-oPRL and an alkaline phosphatase conjugate of goat anti-rabbit IgG. Samples analyzed were (1) total cell protein; (2) soluble portion following cell lysis by a French pressure cell; and (3) pelleted material following cell lysis. The expression vectors were (a) pompPRLF alone; (b) pJL3 and pompPRLF; and (c) pSW1 and pompPRLF. The bPRL standard is 750 ng of pituitary bPRL.

The lack of bPRL secretion from the *E. coli* system was investigated further. One possibility for the lack of soluble bPRL was that high levels of bPRL production may have overwhelmed the ability of the cells to secrete the hormone to the periplasm and thus led to the formation of inclusion bodies. Since YT medium contains components which can induce the lac promoter, growth of the cells in minimal medium (M9+B1) provided a means of more strictly controlling the level of bPRL expression. However, SDS-PAGE and Western blot analysis of the concentrated culture supernatants and the soluble and insoluble fractions of cultures of *E. coli* JM101 containing pompPRLF alone or in combination with pJL3 or pSW1 and grown in minimal medium with the appropriate antibiotics and inducers did not reveal increased amounts of soluble bPRL (data not shown). Another possible reason for the accumulation of insoluble rather than soluble bPRL was the host strain used for expression of the plasmids. The strain used
for secretion of hGH was *E. coli* RV308 (Hsiung et al., 1989); therefore, the bPRL produced from the secretion vectors was compared in strains RV308 and JM101 in order to determine the effect of the host strain on bPRL secretion. An additional difference between the hGH and bPRL secretion systems is that the hGH secretion vector did not contain the fl ori: this was inserted into the plasmid for the bPRL secretion system. Although it seemed unlikely that the presence of the fl intergenic region could affect secretion of bPRL, this possibility was investigated by removing this region thereby creating the vector pompPRL. Duplicate cultures of JM101 and RV308 transformed with pompPRL and pompPRLF were grown in YT + amp for 1.5 h prior to the induction of one culture of each with 2 mM IPTG. Cells in the exponential phase of growth were harvested and fractionated in order to specifically release the periplasmic components (Koshland and Botstein, 1980). Samples from stages in the fractionation procedures were examined for the presence for bPRL by denaturing SDS-PAGE and Western blotting. No differences were observed between strains JM101 and RV308 nor between vectors pompPRL and pompPRLF. The results of the fractionation of RV308 are shown in Figure 11. Two different methods were used to release the periplasmic components: in each case the bPRL remained associated with the insoluble fraction.

### 3.1.3 Intracellular *E. coli* System for Production of Met-bPRL and Met-bPRL Variants

The met-bPRL and met-bPRL variants synthesized in *E. coli* cells are produced as insoluble inclusion bodies and the method developed for the isolation of met-bPRL involves disruption of the cells, centrifugation to pellet the insoluble met-bPRL, and treatment of the pellet with detergents in order to remove contaminating *E. coli* proteins followed by solubilization and renaturation of the met-bPRL. Cells from stationary phase cultures, with a total protein concentration in the range of 0.8 - 1.2 mg/mL (Figures 12A and 13A) were disrupted in the presence of 25 - 50 mM DTT by a single passage at maximum pressure through a French pressure cell. The insoluble material, of which the met-bPRL is a major constituent, was collected by centrifugation and resuspended in buffer containing 5 mM DTT to a protein concentration of approximately 3 - 3.5 mg/mL (Figures 12B and 13B). Treatment of this solution with 0.4% sodium desoxycholate solubilized some *E. coli* proteins (Figures 12C and
A portion of the met-bPRL was solubilized by extraction with 0.1% \(N\)-lauryl sarcosine (sarcosyl) and renatured at pH 10 (Figures 12D and 13D). The protein concentration of the final solutions, consisting primarily of met-bPRLs, was generally in the range of 0.1 - 0.14 mg/mL.

The yield of met-bPRL from the \(E.\) \(coli\) cells was 1 mg from 100 mL of cell culture or less. However, the amount of protein obtained was more than sufficient for the \textit{in vitro} bioassay wherein the ability of the met-bPRL extracts to stimulate the growth of Nb2 cell cultures is determined (Tanaka \textit{et al}., 1980). In addition to providing more than enough recombinant protein for the bioassay, other positive features of the extraction procedure are that it is technically very simple and lends itself well to the processing of many different samples at one time. For bioassay, the hormone preparations were diluted in Fischer's medium containing 10% non-mitogenic horse (gelding) serum and assayed at a number of different concentrations to give a growth response within the useful working range of the assay, \textit{i.e.}, 0.01 - 0.25 ng standard pituitary bPRL/mL.
Figure 11. Western blot analysis of bPRL in fractions from the *E. coli* secretion system. *E. coli* strain RV308 was transformed with the bPRL expression vectors pompPRL or pompPRLF and cultures were grown to stationary phase in YT + amp. Samples obtained following fractionation of the cells to release the periplasmic components were loaded on a denaturing polyacrylamide gel and analysed by a colourimetric Western blot system using polyclonal rabbit anti-oPRL and alkaline phosphatase-linked goat anti-rabbit IgG. Samples analyzed were (1) total cell protein; (2) periplasm control; (3) periplasmic contents; (4) periplasmic membrane components; (5) spheroplast control; (6) periplasmic contents from spheroplasts; and (7) membrane fraction from spheroplasts. (a) vector pompPRL; (b) vector pompPRLF. (+) indicates cells induced with 2 mM IPTG; (-) indicates no added IPTG.
Figure 12. Coomassie blue-stained denaturing polyacrylamide gels of met-bPRL and several met-bPRL variants. The met-bPRLs were synthesized as insoluble inclusion bodies in *E. coli* strain TOPP2 and samples at stages of the bPRL extraction procedure were analyzed under reducing conditions by SDS-PAGE. Samples were (A) total cell protein; (B) pelleted material following lysis of cell cultures by a French pressure cell; (C) insoluble material remaining following extraction of the pelleted material with 0.4% sodium deoxycholate and 0.1% sarcosyl; and (D) renatured sarcosyl extract of the sodium deoxycholate-extracted pellet. Lane 1, unmodified met-bPRL; lane 2, R176A; lane 3, D178E; lane 4, S179A; lane 5, S180A; lane 6, K181A; lane 7, D183A; lane 8, L189A. Variants R176A, D178E, S179A, K181A and L189A were produced efficiently while pESP4 and S180A were synthesized in lesser amounts. Very little of the variant D183A was produced in the TOPP2 cells. The bPRL standard in each case is 500 ng of pituitary bPRL.
Figure 13. Western blot analysis of met-bPRL and several met-bPRL variants. The met-bPRLs synthesized in *E. coli* TOPP2 were extracted with 0.1% sarcosyl from 0.4% sodium desoxycholate-treated insoluble cell material obtained after cell lysis. The sarcosyl-soluble met-bPRL samples were renatured at pH 10. Gels A, B, and C were run under reducing conditions and gel D was run under nonreducing conditions. All samples were analyzed by Western blotting using a chemiluminescent detection system, polyclonal rabbit anti-oPRL, and a horseradish peroxidase conjugate of goat anti-rabbit IgG. Samples were (A) total cell protein; (B) pelleted material following cell lysis; (C) insoluble material remaining following extraction with sodium desoxycholate and sarcosyl; and (D) renatured sarcosyl-soluble met-bPRLs. Lane 1, unmodified met-bPRL; lane 2, R176A; lane 3, D178E; lane 4, S179A; lane 5, S180A; lane 6, K181A; lane 7, D183A; and lane 8, L189A. The bPRL standard in each case is 50 ng of pituitary bPRL. In gel D the faint slow-moving bands in all samples including the standards correspond to high molecular weight aggregates of bPRL, reduced met-bPRL is present in the band slightly above the major band corresponding to oxidized met-bPRL, and the very faint fast-moving band in the met-bPRL samples is most likely a degradation product.
Although bPRL is a monomeric protein (23 kDa molecular weight) high molecular weight aggregates of met-bPRL were often obtained following sarcosyl extraction of transformed E. coli cultures. These aggregates, which react with anti-PRL antibodies, are visible on non-reducing gels as a doublet of bands of approximately 45 - 50 kDa molecular weight along with a number of other less prominent bands of higher molecular weights (Figure 13). The aggregates are presumed to consist of met-bPRL molecules linked covalently through disulfide bonds to itself or to thiol-containing E. coli proteins (Luck and Huyer et al., 1992). Lysis of the E. coli cells in the absence of DTT resulted in the presence of very high amounts of the high molecular weight aggregates in the sarcosyl-soluble extracts. Inclusion of DTT in the buffers used when lysing the cell cultures and isolating the inclusion bodies reduces but does not eliminate the formation of the high molecular weight aggregates (Luck and Huyer et al., 1992). For reasons as yet not understood, the amount of high molecular weight material in the met-bPRL extracts varied between preparations and occasionally a substantial amount of the aggregates was present in spite of the inclusion of DTT. Such preparations were not suitable for quantitation of the hormone and its bioactivity since in addition to being immunoreactive they are probably bioactive. Thus, in this study, the met-bPRLs were quantified for bioassay based on the amount of monomeric oxidized protein present in extracts containing only small amounts of the high molecular weight contaminants. In other experiments, high bioactivity values (in the range of 200% for unmodified met-bPRL) based on the amount of monomeric met-bPRL were obtained from extracts containing large amounts of the high molecular weight aggregates (data not shown). It is not known whether certain of the aggregates stimulated Nb2 cell mitogenesis directly or whether the met-bPRL present in the high molecular weight material dissociated during the course of the bioassay to produce bioactive monomeric bPRL.

3.1.3.1 E. coli Strains for Production of Met-bPRL

As a means of optimizing met-bPRL production in E. coli, a number of different E. coli strains were transformed with the bPRL expression vector pESP4. The level of met-bPRL synthesized in each strain was analyzed by SDS-PAGE and Western blotting as follows. 50 mL and 100 mL YT + amp + 5 mM IPTG cultures of the E. coli strains transformed with pESP4
were grown overnight with shaking at 37°C in 250 mL Erlenmeyer flasks. The insoluble material obtained following lysis of the stationary phase cells was analyzed by denaturing SDS-PAGE. The level of met-bPRL produced varied greatly between the different *E. coli* strains (Figure 14). Very little, if any, met-bPRL was synthesized in strains JM105, RV308, CSH50 or MV1193. The lack of met-bPRL production in strain RV308 is interesting since this strain was used for high-level expression in *E. coli* of the closely related hormone bGH (Schoner *et al.*, 1985). The highest level of met-bPRL expression was obtained in strain Le392F' and the amount of hormone synthesized was slightly higher than in strain HB2151 (data not shown).

The *E. coli* strains TOPP1, TOPP2, and TOPP3, marketed by Stratagene for use in expressing hard-to-express proteins, were also transformed with pESP4 and the level of met-bPRL production examined. Western blot analysis of the renatured sarcosyl-soluble met-bPRL extracts from each of these strains revealed that, amongst the TOPP strains, bPRL production was highest in TOPP2 (Figure 15). Although the amount of met-bPRL synthesized in the TOPP2 cells was less than in Le392F', the amount of high molecular weight aggregates present in the sarcosyl-soluble met-bPRL samples was much less in the TOPP2 extracts than in extracts from the other *E. coli* strains (Figures 15 and 16). For this reason TOPP2 was selected as the host *E. coli* strain for production of met-bPRL.
Figure 14. Coomassie blue-stained denaturing polyacrylamide gels of pelleted material following lysis of various *E. coli* strains transformed with pESP4. *E. coli* strains HB2154, JM105, Le392F, RV308, CSH50, MV1193, NM522, and TG1 were transformed with pESP4 and grown in YT + amp + 5 mM IPTG to stationary phase. The insoluble fraction of each cell culture obtained after cell lysis was analyzed by SDS-PAGE under reducing conditions. The extracts loaded in lane (a) were from 50 mL cultures in 250 mL Erlenmeyer flasks; and in lane (b) from 100 mL cultures in 250 mL Erlenmeyer flasks. The bPRL standard is 500 ng pituitary bPRL.
Figure 15 Western blot analysis of met-bPRL production in *E. coli* TOPP strains 1, 2, and 3. The cell cultures were grown to stationary phase in superbroth (SB) and terrific broth (TB), and the insoluble fraction of each treated with 0.4% sodium deoxycholate and 0.1% sarcosyl. The sarcosyl-soluble met-bPRL samples were renatured at pH 10 and loaded on a nondenaturing polyacrylamide gel for Western blot using a chemiluminescent detection system. The bPRL standard is 50 ng of pituitary bPRL. The very faint slowest-moving anti-oPRL-reactive bands reveal that the high molecular weight met-bPRL aggregates were present in very small amounts. The almost equal intensity for each met-bPRL sample of the band corresponding to the reduced form of bPRL (slower moving) with that of the band corresponding to the oxidized form of bPRL (faster moving) indicates that the renaturation was approximately 60% complete.
Figure 16. Western blot analysis of renatured met-bPRL from various E. coli strains transformed with pESP4. The E. coli strains HB2154, JM105, Le392F, RV308, CSH50, MV1193, NM522, and TG1 were transformed with pESP4 and grown to stationary phase in YT + amp + 5 mM IPTG. Following cell lysis, met-bPRL was isolated by 0.2% sarcosyl-extraction of 1% sodium deoxycholate-treated insoluble material. The sarcosyl-soluble met-bPRLs were renatured at pH 10 and loaded on a non-denaturing polyacrylamide gel for Western blot analysis using a chemiluminescent detection system. Met-bPRL samples in lane (a) are from 50 mL cultures; and in lane (b) from 100 mL cultures. Approximately 20% of each met-bPRL sample was present in the reduced form, as shown by the intensity of the band slightly above that corresponding to the oxidized form of bPRL (indicated with an arrow). The met-bPRL from HB2154 (lane b), Le392F' (lanes a and b), MV1193 (lane b), NM522 (lanes a and b) and TG1 (lanes a and b) contained significant amounts of the high molecular weight aggregates of bPRL, as indicated by the presence of the slowest-moving anti-oPRL-reactive bands. The bPRL standards are the indicated amounts of pituitary bPRL.
3.2 Extraction of Met-bPRL and Met-bPRL Variants from Inclusion Bodies

While the amount of monomeric met-bPRL extracted from E. coli cultures could be increased by the use of higher concentrations of sarcosyl, this also increased the amount of contaminating E. coli proteins (Luck et al., 1989) and of the high molecular weight met-bPRL aggregates. In order to examine the extent to which sarcosyl solubilizes the high molecular weight aggregates, the insoluble material from cultures of E. coli TOPP2 cells expressing unmodified met-bPRL and a number of met-bPRL variants was extracted with 0.4% sodium deoxycholate followed by either 0.1% or 0.2% sarcosyl. The final extracts were loaded on a nondenaturing polyacrylamide gel and analyzed by Western blotting (Figure 17). The figure clearly shows that the amount of high molecular weight aggregates present in the extracts was much lower in the extracts obtained with 0.1% sarcosyl. However, although the proportion of monomeric met-bPRL is higher in the 0.1% sarcosyl extracts the total amount of met-bPRL was much less. It may be noted that the volume of the 0.1% sarcosyl extracts loaded on the gel was double that of the 0.2% extracts.

Differences in the relative amount of hormone extracted by incubation with 0.1% sarcosyl have been observed between met-bPRL variants. In general, a high level of met-bPRL or met-bPRL variant expression in the E. coli cells correlated with a high concentration of hormone in the sarcosyl extract. However, this was not always the case. For example, the amount of the variant Q71V synthesized in E. coli TOPP2 was quite high (Figure 18A, lane 5) but the amount extracted with 0.1% sarcosyl was relatively low (Figure 18B, lane 5). Certain mutations can therefore affect characteristics of the protein other than those relating specifically to function, which in the case of bPRL is the ability to interact with the receptor. Another effect on the solubility of met-bPRL was noted with disulfide bridge variants and with C-terminal truncated mutants. Variants lacking either the C-terminal disulfide bridge or C-terminal residues were almost completely solubilized by treatment with 0.4% sodium deoxycholate (Luck and Huyer et al., 1992; Dr. Luck, Oberlin College, Oberlin, OH, personal communication).
Figure 17. Western blot analysis of met-bPRL and various met-bPRL mutants. Cultures of *E. coli* TOPP2 were grown to stationary phase in terrific broth + amp + 3 mM IPTG. The insoluble material obtained following cell lysis was treated with 0.4% sodium deoxycholate and the met-bPRLs were extracted from the sodium deoxycholate-insoluble material with (A) 0.1% sarcosyl and (B) 0.2% sarcosyl, renatured at pH10, and loaded on a nondenaturing polyacrylamide gel for Western blot analysis using a chemiluminescent detection system. The volume of the 0.1% sarcosyl extracts loaded was twice that of the 0.2% sarcosyl extracts. Lane (1) unmodified met-bPRL; (2) T60A; (3) N170A; (4) H173A; (5) L175A; (6) D178A; (7) Y185A; (8) I193A; and (9) G129R. The amount of hormone extracted varied between samples: T60A and H173A were present in the highest amount while very little D178A was obtained. Renaturation of the met-bPRLs was more than 90% complete as indicated by the very faint band of the reduced form of bPRL visible just above the major band of oxidized bPRL. The slowest-moving anti-oPRL reactive bands correspond to the high molecular weight aggregates of bPRL. The bPRL standards are pituitary bPRL in the amounts indicated.
Figure 18. Coomassie blue-stained denaturing polyacrylamide gels of met-bPRL and various met-bPRL mutants. Cultures of *E. coli* TOPP2 were grown to stationary phase in terrific broth + amp + 5 mM IPTG. The insoluble material obtained following cell lysis was treated with 0.4% sodium desoxycholate and the met-bPRLs were extracted with 0.1% sarcosyl. Samples analyzed by SDS-PAGE were (A) pelleted material following cell lysis; and (B) sarcosyl-soluble met-bPRLs, not renatured at pH 10. Lane (1) unmodified met-bPRL; (2) T60A; (3) L63A; (4) P64A; (5) Q71V; (6) Q73V; (7) Q74V; and (8) E128A. The amount of the met-bPRL variants synthesized (gel A) varied considerably: Q71V was produced at a very high level; T60A, P64A, and E128A were produced at a slightly lower level; unmodified met-bPRL, L63A, and Q73V were synthesized at even lower levels; and no Q74V could be detected. However, the amount of the met-bPRL samples extracted by 0.1% sarcosyl (gel B) showed less variation: approximately equal amounts of all met-bPRL samples except T60A (slightly higher) and Q74V (not detectable) were present in the extracts. The bPRL standard is 500 ng of pituitary bPRL.
3.3 Analysis of Met-bPRL mRNA Levels by Competitive PCR

Mutations may have unexpected secondary effects on gene transcription or on the stability of the protein during synthesis. These effects may be responsible for the poor expression in E. coli of the bPRL variants R125A and D178A. In order to investigate this possibility, reverse transcription was coupled with competitive PCR to quantitate the amount of RNA present in E. coli cells expressing different met-bPRL variants using methods previously described for other systems (Foley et al., 1993; Gilliland et al., 1990; Perrin and Gilliland, 1990).

Competitive PCR was carried out using a single set of primers on the cDNA of interest and a known amount of a competitor DNA. The competitor chosen was the bPRL cDNA containing the signal sequence for the yeast invertase gene: the PCR products from the amplification of the variant bPRL cDNA and the competitor bPRL DNA differed in length by approximately 50 bp and were separable by agarose gel electrophoresis. Comparison of the amount of mRNA present in TOPP2 cells transformed with vectors for poorly expressed mutants (R125A and D178A) with that from cells transformed with more highly expressed variants (unmodified met-bPRL and D178E) did not reveal a significant difference in the relative amounts of mRNA (see Figure 19 for comparison between unmodified met-bPRL and R125A). Therefore, the differences in levels of protein expression did not seem to be related to differences in transcription efficiency.

Poor levels of expression of certain variants could result from inefficient translation or could possibly be a result of decreased protein stability. Thus, substitution of certain residues, particularly those with charged and/or bulky side chains, with a small amino acid such as alanine could destabilize the protein and thus ultimately lead to low levels of expression. Alternatively, problems in mRNA translation could lead to low levels of production of some variants. Codon usage can affect the level of gene expression; therefore, use of E. coli preferred codons often improves expression of heterologous proteins (Gouy and Gautier, 1982; Grosjean and Fiers, 1982; Robinson et al., 1984). A single amino acid can be represented by any one of several codons but the frequency with which each synonymous codon is used is not identical. More frequently used codons may correspond to more frequent tRNAs and thus the high translation efficiency seen with the genes for highly expressed proteins may be a consequence of bias
towards the most common codons. However, codon bias is not absolute and it is therefore
difficult to predict which codon at any one position will lead to efficient expression. Thus, while
mutations in the bPRL cDNA were designed to incorporate the more frequently used codons,
certain choices may have deleteriously affected gene translation.

Figure 19. Quantitation of RNA by competitive PCR. cDNA prepared using AMV reverse
transcriptase from total RNA from E. coli TOPP2 expressing (A) R125A and (B) unmodified
met-bPRL was amplified in the presence of a dilution series of pMH4 DNA (bPRL cDNA fused
to the signal sequence from the yeast invertase gene). The flanking primers were: MH53, 5'-
GTGAGCGGATATTTCCACACAGGA-3'; and MH19, 5'-TCTCAGAAATGGATAG-
GATCCCAATGTG-3'. Following 25 rounds of PCR amplification samples loaded on a 1.9%
MetaPhor agarose gel in TBE buffer were visualized by UV light following staining with
ethidium bromide (0.5 μg/mL in dH2O). Lanes 1 - 10 are from amplification reactions
containing pMH4 plasmid DNA ranging in the relative amounts: 100, 30, 20, 8, 4, 1.2, 0.8, 0.32,
0.16, and 0.016 (reaction 1 contained approximately 5 amol pMH4). Controls are amplification
reactions carried out in the absence of competitor DNA on (A) pMH1 DNA; (B) pMH4 DNA;
(C) TOPP2 cDNA; and (D) dH2O.
3.4 Renaturation of Met-bPRL and Met-bPRL Variants

In previous studies it was found that met-bPRL and met-bPRL variants extracted from insoluble inclusion bodies by consecutive extractions with 1% sodium desoxycholate and 0.2% sarcosyl was soluble, but exhibited only about 40% of the bioactivity of the native hormone. A slight increase in the bioactivity of sarcosyl extracts of met-bPRL was noted to occur spontaneously during storage of the samples at 4°C although fully bioactive protein was not obtained (Luck et al., 1989). Western blot analysis of met-bPRL extracts electrophoresed on a nondenaturing acrylamide gel revealed bands corresponding to the reduced and oxidized forms of bPRL, as can be seen in Figure 20 (Luck and Huyer et al., 1992). The low level of bioactivity was therefore felt to be due to problems in disulfide bond formation, as bPRL contains three disulfide bridges: incorrect and incomplete disulfide bond formation can cause proteins to fail to attain the correct conformation and therefore form inclusion bodies (Pigiet and Schuster, 1986; Schoemaker et al., 1985). Exposure of the samples to air may have promoted correct disulfide bond formation between some of the initially reduced cysteine residues but would not have caused incorrectly formed disulfide bonds to break. In this study, renaturation of met-bPRL was found to be also promoted by the growth medium used for the Nb2 cell bioassay. Thus, extracts of met-bPRL incubated with Fischer's medium containing β-mercaptoethanol in a CO2-enriched atmosphere over a 48 h period exhibited an increase in the amount of oxidized met-bPRL and a corresponding decrease in the quantity of reduced protein (Figure 20).

The refolding of proteins obtained from inclusion bodies is generally accomplished by removing the denaturing agent, usually by dialysis or dilution, and is influenced by a number of different variables (Marston, 1987). Factors such as the rate at which the denaturing agent is removed, the concentration and purity of the protein preparation, and the pH affect the yield of active protein. Methods which have been used successfully to obtain active PRL include dialysis of inclusion body material solubilized by 8 M urea (Gilbert et al., 1991; Paris et al., 1990) and incubation at high pH (Dr. Byatt, Monsanto Corp., St. Louis, MS, personal communication). The method initially used to convert the sarcosyl-soluble met-bPRL to a fully active form was air incubation with a 50-fold excess of thioredoxin used as a 10:1 redox mixture of the oxidized and
Figure 20. Western blot analysis of non-renatured met-bPRL incubated in Nb2 cell growth medium. Met-bPRL was extracted using 0.4\% sodium desoxycholate and 0.2\% sarcosyl from *E. coli* TOPP2 transformed with pESP4. The sarcosyl-solubilized met-bPRL was diluted 1:50 with Fischer's medium containing 10\% gelding serum and 0.1 mM \( \beta \)-mercaptoethanol. The approximate final concentration of hormone was 0.5 \( \mu \text{g/mL} \). Samples were sparged with 5\% CO\(_2\)/air, incubated at 37°C for the indicated amounts of time, loaded on a non-denaturing polyacrylamide gel, and analyzed by Western blotting using a chemiluminescent system, polyclonal rabbit anti-oPRL, and a horseradish peroxidase conjugate of goat anti-rabbit IgG. The pH of all samples remained at pH 7.4 during incubation. Control samples were (a) met-bPRL in 0.1 M sodium phosphate buffer, pH 7.4; (b) met-bPRL in Fischer's medium containing gelding serum but not sparged with 5\% CO\(_2\); and (c) met-bPRL in Fischer's medium containing gelding serum and \( \beta \)-mercaptoethanol and sparged with 5\% CO\(_2\). The slow-moving bPRL-reactive bands indicates that significant amounts of high molecular weight aggregates of bPRL were present in all samples. The bPRL standards are the indicated amounts of pituitary bPRL.

reduced forms (Luck *et al.*, 1989). However, thioredoxin is not an ideal renaturing agent because it is expensive and its use leads to the introduction of a large amount of foreign protein into the samples. Therefore, an alternative method of renaturing met-bPRL and met-bPRL variants by incubating the sarcosyl-solubilized met-bPRL extracts at pH 10 in the presence of air was developed (Luck and Huyer *et al.*, 1992). This procedure generally resulted in the efficient and rapid refolding of the hormone. However, occasionally incubation at pH 10 did not lead to complete renaturation of the met-bPRL. In this study, attempts to determine the source of the
variability in the degree of renaturation focussed on the length of time of sample incubation at pH 10, the volume and concentration of each sample, the amount of air introduced to the samples, and the effect of different concentrations of sodium deoxycholate and sarcosyl (data not shown). No reason was found for the variability.

3.5 Structure-Function Analysis of Met-bPRL

Bovine PRL is a 23 kDa globular protein containing three disulfide bridges and four putative α-helices (Figure 21) and although no three-dimensional structure has been established, it is generally anticipated that the structure of bPRL is similar to that of growth hormone (see Introduction). In an effort to determine the relationship between amino acid residues and the mitogenic activity of met-bPRL site-directed mutagenesis was used to examine the effect of changes of selected amino acids on the mitogenic activity of the hormone in the Nb2 cell assay. It has previously been shown that the mitogenic activity of met-bPRL is equivalent to that of native bPRL (Luck et al., 1989). The amino acid changes involved the substitution, primarily by alanine, of bPRL residues which either corresponded directly to those involved in receptor interactions in related hormones or which fell within the specific regions of the hormone that, based on structural homologies with hGH, are postulated to play a role in contacting the lactogenic receptor.

Figure 21. Diagram of bPRL showing its three disulfide bridges and the locations of the putative helical regions. Numbers indicate the position of the cysteine residues and the putative limits of the helical regions.
3.5.1 Putative Lactogen-Specific Residues Identified Via Comparative Studies

An initial study of the relationship between the structure of met-bPRL and its mitogenic activity in the Nb2 cell assay focussed on seven residues conserved amongst a group of 10 pituitary lactogens (bovine, equine, human, mouse, ovine, porcine, rat, and whale PRLs, and human and monkey GHs) that are not found in the related, but non-lactogenic bGH (Luck et al., 1989 and Figure 22). Variants of met-bPRL which had been made by substituting in met-bPRL corresponding bGH amino acids had bioactivities of the same order as that of unmodified met-bPRL. However, although this strategy of substituting a conserved amino acid from a lactogenic hormone with the corresponding residue from a non-lactogenic growth hormone failed to distinguish any lactogen-specific amino acids in bPRL, a similar approach was used successfully to identify three residues which play a role in the mitogenic activity of mPL-II (Davis and Linzer, 1989b). In the case of mPL-II, five residues common to a group of lactogenic hormones that differed at the corresponding positions in a group of nonlactogenic hormones were altered to the residues in the nonlactogenic hormone proliferin.

A comparison of the sequences of bPRL and mPL-II indicated that residues R21, R177, and K187 of bPRL are equivalent to the mPL-II residues identified as being critically important for binding to the PRL receptor and for PRL-induced mitogenesis of Nb2 cells (namely R14, R169, and K179) (Figure 22). The importance of the equivalent three bPRL residues to the mitogenic activity of the hormone was investigated (Luck and Huyer et al., 1991). Each of these three residues was substituted with alanine, asparagine, leucine, and lysine or arginine. Alanine, a small, nonpolar residue, was chosen for substitution because it can be perceived to be the most innocuous replacement (Cunningham and Wells, 1989) while asparagine and lysine or arginine were chosen because these residues are in the equivalent positions to R21, R177, and K187 of bPRL in the non-lactogenic bGH. Leucine was chosen because the R14L mPL-II variant showed a large decrease in mitogenic activity (Davis and Linzer, 1989b). The mitogenic activities of the variants are shown in Table 4. Changes of any of these three residues to alanine, leucine, or asparagine resulted in decreases in mitogenic activity while an effect from the more conservative interchange of arginine and lysine was seen only at position 177. The importance of R177 to the
mitogenic activity of bPRL was examined further through a number of additional substitutions. Replacement of this residue with aspartate, glutamate, tyrosine, glycine, serine, glutamine, histidine, and phenylalanine resulted in variants all with very low bioactivity (≤ 5%) (Luck and Huyer et al., 1991).

Figure 22. The complete amino acid sequences of the lactogenic hormones bPRL, hPRL, mPL-II, and hGH and of the related but non-lactogenic bGH. The sequences are aligned to maximize the homology between the hormones (Luck et al., 1989). The numbers refer to the amino acid locations in bPRL. The underlined residues are conserved in eight mammalian pituitary PRLs (bovine, equine, human, mouse, ovine, porcine, rat, and whale). The seven residues found in 10 pituitary hormones (bovine, equine, human, mouse, ovine, porcine, rat, and whale PRLs plus human and monkey GHs) but not in bGH are marked by asterisks (*).
Table 4. Mitogenic Activities of Met-bPRL Variants Produced by Amino Acid Substitutions at Positions R21, R177, and K187

<table>
<thead>
<tr>
<th>Amino Acid Substitution</th>
<th>% Activity Relative to Pituitary bPRL(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified met-bPRL</td>
<td>101.5 ± 5.0</td>
</tr>
<tr>
<td>R21A</td>
<td>20.9 ± 0.2</td>
</tr>
<tr>
<td>R21L</td>
<td>29.2 ± 0.2</td>
</tr>
<tr>
<td>R21N</td>
<td>50.0 ± 4.0</td>
</tr>
<tr>
<td>R21K</td>
<td>88.6 ± 5.6</td>
</tr>
<tr>
<td>R177A</td>
<td>1.1 ± 0.05</td>
</tr>
<tr>
<td>R177L</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>R177N</td>
<td>2.4 ± 0.03</td>
</tr>
<tr>
<td>R177K</td>
<td>12.8 ± 1.6</td>
</tr>
<tr>
<td>K187A</td>
<td>46.3 ± 7.3</td>
</tr>
<tr>
<td>K187L</td>
<td>41.8 ± 8.8</td>
</tr>
<tr>
<td>K187N</td>
<td>81.0 ± 2.0</td>
</tr>
<tr>
<td>K187R</td>
<td>99.1 ± 4.9</td>
</tr>
</tbody>
</table>

\(^a\) The data obtained for each variant are the means (±SEM) of the results of bioassays done with three different mutant met-bPRL preparations.

3.5.2 Substitutions of Residues in the Loop Region Between Putative Helices 1 and 2

Investigations into the interaction of hGH with the hGHbp and the hPRLbp have revealed that residues within the second half of the loop joining helices 1 and 2 are involved in binding of the hormone to these receptors (Cunningham and Wells, 1989, 1991). In addition, this region was shown to be important to the mitogenic activity of hPRL (Goffin et al., 1992). Although these amino acids (comprising residues 58 - 74 in bPRL) do not, in any of the related hormones that have been examined (Figure 22), appear to form part of a specific structural element such as a helix or β-sheet, the sequence of this segment is highly conserved within the hormone family suggesting that for all of these proteins this region is structurally and/or functionally important (Goffin et al., 1992).

The importance of this putative loop region to the mitogenic activity of bPRL was investigated through alanine-scanning mutagenesis. Met-bPRL variants, each containing a single
mutation, were produced as described in Materials and Methods. Residues T60, S61, L63, P64, P66, E67, and K69 were each replaced by alanine. Residues Q71, Q73, and Q74 were each replaced by alanine and valine and H59 was replaced by alanine and serine. The contributions of the amino acids S62, T65, D68, and E70 to the mitogenic activity of bPRL were examined in an earlier study: substitutions at any of these positions had only a minor effect on activity (Luck et al., 1989). Position 72 was not examined because it already contains an alanine. All of the variants were produced as insoluble inclusion bodies in E. coli TOPP2 cells except the Q71, Q73, or Q74 variants which were produced in HB2151 cells; they were solubilized with 0.1% sarcosyl and renatured by air oxidation at pH 10. Western blot analysis of the variants subjected to polyacrylamide gel electrophoresis under non-reducing conditions revealed that greater than 80% of each preparation had the same electrophoretic mobility as pituitary bPRL, thus indicating that each had renatured with the C58-C174 disulfide bond intact (Luck and Huyer et al., 1992).

Most of the met-bPRL variants were expressed in E. coli strain TOPP2 cells at a level comparable to that of unmodified met-bPRL. The low levels of expression observed when Q71, Q73, and Q74 were substituted led to difficulties in extracting sufficient quantities of the hormone for the Nb2 cell bioassay. In fact, the variants with alanine substitutions at these positions were produced at such low levels in the TOPP2 cells that their mitogenic activity could not be determined. Expression of the Q71, Q73, and Q74 variants, containing either alanine or valine substitutions, was much higher in E. coli strain HB2151 (data not shown) and bioactivity values were determined for hormone extracted from these cells.

The results of the bioassays on these variants are presented in Figure 23. Variants T60A (72%), S61A (71%), P66A (70%) and E67A (90%) were nearly as bioactive as unmodified met-bPRL while the mitogenic activity of L63A (51%) and P64A (61%) was slightly reduced. H59A (38%), H59S (23%), Q71V (35%), Q73V (38%), and Q74V (36%) each showed an approximate three-fold reduction of Nb2 cell activity whereas the K69A variant (151%) exhibited a bioactivity 1.5-fold greater than that of unmodified met-bPRL. The bioactivities of the variants carrying alanine substitutions at Q71, Q73, and Q74 (48%, 45%, and 44%, respectively) were determined using a single extract of each mutant.
Figure 23. Bioactivities of met-bPRL variants obtained by single alanine substitutions of met-bPRL residues within the loop region joining putative helices 1 and 2. The bioactivities were determined using the Nb2 cell assay and are expressed as a percentage relative to pituitary bPRL (average ±SEM). Three to five different extracts of each mutant (except one extract of Q74V) were assayed and each extract was assayed over a range of concentrations. No error bar is shown for Q74V because only one extract of this variant was assayed for mitogenic activity. For simplicity, the mitogenic activities of H59S (23%), Q71A (48%), Q73A (45%), and Q74A (44%) are not included in the Figure.
3.5.3 Substitution of Met-bPRL Residues in Putative Helix 4

The importance of residues within the fourth α-helix of hGH for binding to both the somatogenic and lactogenic receptors has been established through a series of alanine-scanning mutagenesis studies (Cunningham and Wells, 1989, 1991) and confirmed by analysis of the crystal structures for the hGH-(hGHbp)_2 complex (de Vos et al., 1992) and the hGH-hPRLbp complex (Somers et al., 1994). Although the binding site of PRL to the lactogenic receptor is assumed to be very similar to that described for hGH, few studies have been carried out to determine the involvement of helix 4 residues in PRLs in their interaction with the receptor.

In this study the contribution of residues in the C-terminal two-thirds of helix 4 to the mitogenic activity of bPRL was investigated by alanine-scanning mutagenesis. Met-bPRL variants, each containing a single substitution, were prepared and the mitogenic activity of each variant measured using the Nb2 cell bioassay system. Residues N170, L171, H173, L175, R176, S179, S180, D183, T184, Y185, L188, L189, R192, and I193 were all replaced with alanine, D178 was replaced with glutamate and serine, and K181 was replaced by alanine, leucine, and arginine. Residues C174 and C191 were investigated in a previous study which showed that the mitogenic activity of the C191S variant was equivalent to that of unmodified met-bPRL while essentially no activity could be detected with the C174S variant (Luck and Huyer et al., 1992). However, the effect of this latter mutation is believed to be due to disruption of the C58-C174 disulfide bond which is critical for maintaining the three-dimensional structure of bPRL. While R177 is very important, K187 does not play a significant role in the mitogenic activity (section 3.5.1 and Luck and Huyer et al., 1991). Residues L172, I182, L186, and N190 were not examined because they are on the hydrophobic side of the putative helix and are therefore in the interior of the hormone molecule and unlikely to interact with the receptor (Figure 24).

All of the variants were produced as insoluble inclusion bodies in TOPP2 cells, solubilized with 0.1% sarcosyl, and renatured by air oxidation at pH 10. All the mutant hormones were expressed at levels similar to or greater than unmodified met-bPRL, except for those containing substitutions at positions D178 and D183 which were produced in much lower amounts. However, while the level of expression of D178A was so low that sufficient protein could not be
extracted for analysis in the Nb2 cell bioassay, enough of the more conservative mutants D178E and D178S was produced to permit a determination of the contribution of this residue to the mitogenic activity. Typical Western blots of some of the bPRL variants, made from gels run under non-reducing conditions, are shown in Figures 25 and 26. Approximately 80% of each met-bPRL preparation had the same electrophoretic mobility as pituitary bPRL, indicating almost complete renaturation of the hormones.

Figure 24. Helical wheel diagram for putative helix 4 of bPRL. The helix is strongly amphipathic, as shown by the distribution of charged (filled symbols), polar but uncharged (shaded symbols), and hydrophobic (open symbols) residues.
Figure 25. Western blot analysis of sarcosyl extracts of met-bPRL and various met-bPRL mutants. The met-bPRLs were extracted with 0.1% sarcosyl from 0.4% sodium deoxycholate-treated insoluble material from transformed E. coli TOPP2 cells. The sarcosyl-soluble met-bPRL samples were loaded on a nondenaturing polyacrylamide gel and analyzed by Western blotting using a chemiluminescent detection system. Lane (1) unmodified met-bPRL; (2) T60A; (3) E128A; (4) N170A; and (5) L175A. The major band corresponds to oxidized hormone, the faint band slightly above the major band corresponds to the reduced form of the hormone and the very faint slow-moving bands are high molecular weight immunoreactive aggregates. The bPRL standards are pituitary bPRL in the amounts indicated.

Figure 26. Western blot analysis of sarcosyl extracts of various met-bPRL mutants. The met-bPRLs were extracted with 0.1% sarcosyl from 0.4% sodium deoxycholate-treated insoluble material from transformed E. coli TOPP2 cells. Samples were loaded on a nondenaturing polyacrylamide gel and analyzed by Western blotting using a chemiluminescent detection system. Lane (1) S179A; (2) D178S; (3) D178E; (4) R176A; and (5) S180A. The major band represents the oxidized, renatured form of the hormone, the faint band above the major band corresponds to the reduced form of the hormone and the very faint slow-moving bands correspond to high molecular weight immunoreactive aggregates. The bPRL standards are pituitary bPRL in the amounts indicated.
The mitogenic activities of each putative helix 4 met-bPRL variant are shown in Figure 27. Variants T184A (92%), L189A (84%), and R192A (94%) were essentially as bioactive as unmodified met-bPRL. In addition, little effect resulted from the alanine replacements of H173 (58%), L175 (58%), S179 (64%), S180 (53%), Y185 (66%), L188 (66%) and I193 (62%). An approximate three-fold decrease in mitogenic activity was observed with the recombinant proteins L171A (42%), R176A (32%), and D183A (36%). The greatest effect on bioactivity was obtained with the variants containing substitutions at positions 178 (serine and glutamate replacements both gave mitogenic activities of 9%) and 181 (the alanine and leucine variants had 5% and 4% activity, respectively). From these data it therefore appears that D178 and K181, in addition to the previously identified R177 (section 3.5.1) play very important roles in the mitogenic activity of met-bPRL. Of the other 11 residues examined only replacements of L171, R176, and D183 seem to affect the interaction of bPRL with the Nb2 receptor, albeit to a much lesser extent than replacements of D178, R177, or K181.

3.5.4 Substitution of Met-bPRL Residues in the Putative Site 2 Binding Site

Human GH contains two binding sites for the hGHbp: the hormone first binds a receptor molecule at site 1 and then a second hGHbp is bound at site 2 (Cunningham et al., 1991; de Vos et al., 1992). The residues comprising site 2 are located at the N-terminus of the hGH molecule and in the middle of the third helix. Although definitive proof of receptor dimerization by other members of the growth hormone/prolactin/placental lactogen family has not been reported it is anticipated that this mechanism of receptor binding is common to this family of hormones.

The present study of bPRL, using the Nb2 cell assay, should be able to detect residues critical for site 1 and site 2 function. Therefore, some residues equivalent to those which in hGH affect site 2 binding were examined. Amino acid replacements were performed at R125, E128, and G129 of bPRL. Unfortunately, while the E128A variant was synthesized in TOPP2 cells at a level equal to or exceeding that of unmodified met-bPRL (Figure 18) the amount of R125A produced was so low that the contribution of this residue to the mitogenic activity of bPRL could not be determined. The bioactivity of E128A was 44% that of unmodified met-bPRL while substitution of G129 with arginine resulted in a variant with no activity in the Nb2 cell assay.
Figure 27. Bioactivities of met-bPRL variants with single amino acid substitutions in putative helix 4 residues. The bioactivities were determined using the Nb2 cell assay and are expressed as a percentage relative to the bioactivity of pituitary bPRL (average ±SEM). Three to six different extracts of each mutant were used and each extract was assayed over a range of concentrations.
DISCUSSION

4.1 Production of Bovine Prolactin

Many different factors are involved in the successful intracellular production of soluble and active proteins. In the bacterial and eukaryotic cell a complex process involving the protein, chaperones, co-chaperones, and foldases determines whether the protein will accumulate in the cytoplasm as the native structure or in aggregates (reviewed by Gilbert, 1994). However, many folding mechanisms exist and different proteins behave differently in each system: a set of conditions allowing the production of one protein in native form will not necessarily result in production of another protein in its natural form. Further, since each protein requires its own combination of chaperones and foldases, achieving this in heterologous systems is obviously difficult.

In contrast, the production of proteins secreted in the periplasm of *E. coli* is fairly well established as a means of generating recombinant proteins (reviewed in Blight *et al.*, 1994) and offers many advantages over cytoplasmic production. The greatest advantage is that of producing a correctly folded and soluble protein. Efficient production and secretion of hGH into the *E. coli* periplasm has been achieved by fusing the hGH gene to a signal sequence from an *E. coli* gene, in particular that from alkaline phosphatase (*phoA*) (Gray *et al.*, 1985), that from heat-stable-enterotoxin II (*STII*) (Chang *et al.*, 1987; Chang *et al.*, 1989), or that from the outer membrane protein A (*ompA*) (Becker and Hsiung, 1986; Hsiung *et al.*, 1986). The signal sequence aids in the translocation of proteins across the cytoplasmic membrane and cleavage by a signal peptidase then releases the mature protein into the periplasmic space (Inouye *et al.*, 1984). The hGH produced in these systems was processed properly, had formed the correct disulfide bonds, and appeared to have a secondary structure identical to that of native hGH.

Secretion to the periplasmic space of *E. coli* is thus generally more convenient than cytoplasmic production, particularly when the latter results in the formation of insoluble inclusion bodies. However, a system wherein the foreign protein is released to the culture medium offers even more advantages. In addition to obtaining a correctly folded and soluble
protein the medium provides a larger volume for accumulation of the foreign protein and contains few proteases which could degrade the protein. As well, the absence of many other proteins in the medium simplifies the purification of the protein of interest. Two systems for secretion of hGH to the culture medium of E. coli have been reported (Hsiung et al., 1989; Kato et al., 1987). In one system the hGH expression vector carried the weakly activated kil gene, the penicillinase promoter, and the hGH gene fused to a signal sequence from an alkalophilic Bacillus sp. The hGH was secreted to the periplasm and then released to the culture medium by the action of the kil gene which permeabilized the outer membrane (Kato et al., 1987). In the other system secretion of hGH to the culture medium of E. coli was obtained by refining a system designed to export hGH to the periplasm (Hsiung et al., 1989). The gene for the bacteriocin release protein (BRP) was cloned into a vector and placed under the inducible control of the lpp-lac promoter-operator system. BRP activates the detergent-resistant phospholipase A of the outer membrane and thus causes the membranes to be permeabilized. Low levels of expression of the BRP, achieved by controlling the level of the inducer IPTG, resulted in the specific release of proteins from the periplasmic space to the culture medium. Thus, transformation of E. coli with a vector containing hGH fused to the ompA signal sequence along with a BRP expression vector resulted in efficient secretion of hGH to the medium (Hsiung et al., 1989).

As described above, there has been some success in producing soluble hGH from E. coli; however, in spite of the homologies between GH and PRL, PRL is much more commonly produced as insoluble inclusion bodies. Attempts in this study to obtain soluble, secreted bPRL from S. cerevisiae and from E. coli were unsuccessful: no hormone could be detected in the yeast containing the bPRL expression vector in which the bPRL gene was fused to the yeast invertase signal sequence, and all bPRL produced in the E. coli cells was found associated with the insoluble cell fraction. The reason for the lack of bPRL production in S. cerevisiae is unknown. The E. coli secretion system, derived from the dual vector system for hGH secretion described above, in which a vector containing the bPRL gene fused to the ompA signal sequence was co-transformed into E. coli with a vector for expression of the bacteriocin release protein, did not
produce soluble bPRL. This result is puzzling since this system was used successfully for secretion of the related hormone hGH.

Only a few examples of expression of biologically active PRL have been reported. Soluble chicken PRL (chPRL) was produced in E. coli; however, although the level of expression of chPRL was approximately 1.5% of total cell protein, 95% of the chPRL accumulated in inclusion bodies and only 2 - 3% could be isolated as the soluble and biologically active form (Hanks et al., 1989). Efficient production of biologically active hPRL was obtained in E. coli using a high-expression vector containing a chimeric gene encoding a fusion of protein A and hPRL (Hiraoka et al., 1991). The amount of hormone produced was 0.06 - 0.2% of total E. coli protein; however, approximately 60% of the hPRL molecules were inactive, possibly as a result of incorrect disulfide bond formation. In addition, the hPRL obtained after digestion with collagenase to remove the protein A component had an additional four amino acids at the N-terminus, a tag resistant to collagenase action. Periplasmic secretion of a biologically active variant of hPRL has recently been obtained (Morganti, L., Huyer, M., Gout, P.W. and Bartolini, P. 1995. Production and Characterization of Biologically Active Ala-Ser-(His)_{6}-Ile-Glu-Gly-Arg-Human Prolactin, Secreted in the Periplasmic Space of Escherichia coli. Manuscript submitted). Secretion of hPRL to the periplasm was obtained using a leader sequence from the cellulase gene of Cellulomonas fimi. The hPRL produced contained a 12 amino acid peptide tag at the N-terminus but had biological activity of the same order as pituitary hPRL. Other reports of bacterial production of PRL describe the accumulation of the hormone in inclusion bodies (Cunningham et al., 1990; Gilbert et al., 1991; Luck et al., 1986, 1989; Paris et al., 1990). Production of soluble, biologically active, authentic PRL has not yet been reported. Although in animals PRL is produced in soluble form and is naturally secreted, further work needs to be carried out to determine the conditions necessary to obtain soluble bacterially-produced PRL.

As in the case for bPRL, the high-level expression of eukaryotic proteins in E. coli often results in the formation of insoluble inclusion bodies which can accumulate to over 20% of the total E. coli cell protein (reviewed in Mitraki and King, 1989). Inclusion bodies are amorphous non-native protein aggregates clearly separated from the rest of the cytoplasm, but not
surrounded by a membrane (Schoemaker et al., 1985; Schoner et al., 1985; Williams et al., 1982) and they remain particulate after the cells are broken open. Inclusion body formation is believed to be due to the specific association of partially folded intermediates rather than to the precipitation of native proteins synthesized at levels above their solubility. Thus, production of the aggregates is affected by environmental conditions such as temperature and by the presence or absence of factors such as chaperones during the maturation process (Mitraki and King, 1989).

Although the aggregates are not composed of correctly folded protein, there are positive features to the production of protein in inclusion bodies. These advantages include the high levels of protein expression generally obtained and the relative ease of isolating fairly pure protein since the E. coli cytoplasmic proteins can be easily washed away. However, there are also a number of disadvantages associated with inclusion bodies, the most notable amongst these being the difficulty in converting the insoluble, aggregated heterologous protein to a soluble and active form. A number of agents can be used to solubilize inclusion bodies including denaturants such as urea and guanidinium chloride, detergents, organic solvents, and extremes of pH (Marston, 1987). These agents disrupt the ionic and/or hydrophobic interactions between the polypeptide chains and result in denaturation of the protein. Thus, a renaturation step is necessary in order to obtain active protein.

The procedure for extracting met-bPRL from the insoluble fraction of E. coli cells transformed with the bPRL expression vector involves mechanical lysis of the cells using a French pressure cell, centrifugation to harvest the insoluble met-bPRL, treatment with sodium desoxycholate to solubilize some contaminating E. coli proteins, and extraction with sarcosyl to solubilize the met-bPRL. Fully active met-bPRL is not obtained from this procedure (Luck et al., 1989); therefore, the sarcosyl-soluble met-bPRL was renatured by air oxidation at pH 10 (Luck and Huyer et al., 1992). The renaturation was assessed by Western blot analysis of the samples run under non-denaturing conditions: the oxidized form of bPRL, containing the C58-C174 disulfide necessary for biological activity, is more compact than the reduced form; thus, these two forms can be distinguished on a non-denaturing polyacrylamide gel. Although the majority of the met-bPRL obtained by this procedure is in the monomeric and oxidized form, a
portion of the met-bPRL is usually present as high molecular weight aggregates, presumably consisting of the hormone met-bPRL covalently linked via disulfide bonds to itself or to thiol-containing *E. coli* proteins (Luck and Huyer *et al*., 1992).

In previous studies, *E. coli* HB2151 was used as the host strain for the met-bPRL expression vector pESP4 and transformed cells were grown in YT medium (Luck *et al*., 1989, 1990, 1991, 1992). In this study, in an attempt to optimize production of met-bPRL, a number of different *E. coli* strains were transformed with pESP4 and cultures were grown in terrific broth, a richer medium than YT. Production of met-bPRL varied greatly among different *E. coli* strains, ranging from very little or no production in strains JM105 and CSH50 to high levels of production in strains Le392F', NM522, and TOPP2 (Figures 14, 15, and 16). Although the total amount of hormone synthesized in the TOPP2 strain was not maximal, this strain was chosen for routine production because the amount of high molecular weight met-bPRL aggregates present was lower in TOPP2 cell extracts than in those from any of the other *E. coli* strains tested (Figures 15 and 16). The use of 0.1% sarcosyl, rather than the 0.2% sarcosyl used in earlier studies, to extract the met-bPRL from the *E. coli* extracts was also found to decrease the proportion of the high molecular weight aggregates in the met-bPRL samples and 0.1% sarcosyl was therefore used for routine extraction.

*E. coli* expression systems are often chosen for the production of recombinant proteins since, in addition to the ease of manipulating *E. coli*, the vast amount of knowledge that has been obtained about this organism has led to the engineering of a number of different vectors and strains which can be used to achieve efficient and controlled synthesis of almost any gene product (Das, 1990). However, there are disadvantages to expression of heterologous proteins in *E. coli*. Some eukaryotic proteins produced in *E. coli* are toxic to the cell and are degraded on synthesis; others could only be produced as fusion proteins of which none could be processed appropriately. In addition, a common consequence of high levels of expression is the aggregation of the protein in insoluble inclusion bodies. Although the latter problem is not limited to *E. coli*, eukaryotic expression systems can be used to circumvent some of the problems encountered with the bacterial systems (Bradley, 1990).
Eukaryotic expression systems used successfully for the production of eukaryotic proteins include expression in yeast, in mammalian cells, and in insect cells using baculovirus vectors (Ausubel et al., 1994; Bradley, 1990). Advantages of these expression systems are that the recombinant proteins are generally properly processed and usually end up in the correct cellular compartment. Disadvantages of these systems include greater complexity, cost, and longer processing times. However, in spite of these drawbacks the use of eukaryotic expression systems for the production of eukaryotic proteins is becoming more common and more popular, and as the knowledge about, and familiarity with these systems grows, the common problems become easier to handle. At the time that the expression system for bPRL was developed (Luck et al., 1986) E. coli systems were by far the preferred routes for production of recombinant proteins. Indeed, bacterial systems, as well as yeast systems, are still often preferred when the intention is to create and analyze many different mutants. However, given the problems that have been encountered in the production of active met-bPRL from E. coli and the advances that have been made in the development of eukaryotic expression systems, investigation of the latter systems for the synthesis of bPRL would likely be worthwhile.

4.2 Structure-Function Analysis of Met-bPRL

The relationship between the primary structure and biological activity of bPRL was investigated by determining the effects of specific amino acid changes on the mitogenic activity of the hormone. All amino acid changes were produced by site-directed mutagenesis, and, for the most part, involved the substitution by alanine of residues corresponding to those which either corresponded directly to those involved in receptor interactions in related hormones or which fell within the specific regions of the hormone that, based on structural homologies with hGH, are postulated to play a role in contacting the receptor.

The mitogenic activity of bPRL was significantly decreased by replacement of the arginine at position 177 with any one of 12 different amino acids, suggesting that this residue is very important for the biological activity of the hormone. The reduced mitogenic activities resulting from replacement of residue R21 suggests that this residue, whilst not essential, may also be
involved in the activity of bPRL. However, since substitution of this arginine with the
functionally similar residue lysine had little effect on the mitogenic activity of bPRL (activity of
R21K was 87% that of unmodified met-bPRL), the activity of the hormone is not as dependent
on an arginine at position 21 as it is on one at position 177. In contrast, the residue K187 does
not appear to play an important role in the mitogenic activity of the hormone: no substitutions at
this position severely affected the bioactivity of bPRL.

A study of the lactogenic hormone mPL-II had revealed that three lactogen-specific
residues (R14, R169, and K179) play significant roles in the mitogenic activity of this hormone
in the Nb2 cell bioassay system and in the binding of mPL-II to the Nb2 PRL receptor (Davis
and Linzer, 1989b). However, an examination of the roles of the corresponding bPRL residues
(R21, R177, and K187) in the mitogenic activity of this hormone revealed differences from
mPL-II in its response to mutational changes at these positions (Luck and Huyer et al., 1991).
Whereas all of the mPL-II variants (R14L, R167N, and K179N) had mitogenic activities in the
Nb2 cell assay that were 20- to 30-fold lower relative to wild-type mPL-II; of the corresponding
bPRL variants (R21L, R177N, and K187N) only one, R177N, had very low mitogenic activity
(2.4% relative to unmodified met-bPRL). The substitution at position 21 resulted in a much
lesser decrease in mitogenic activity (29% for R21L), and the mitogenic activity of variant
K187N (87%) was close to that of unmodified met-bPRL (Table 4). Other substitutions at these
positions resulted in somewhat similar decreases in mitogenic activity, indicating that of the
three residues found to have important roles in the mitogenic activity of mPL-II, only the bPRL
residue corresponding to R167 of mPL-II (namely R177) plays an equally important role in the
mitogenic activity of met-bPRL. Interestingly, the hGH residues equivalent to R177 and K187
of bPRL (K168 and R178, respectively) have both been shown to be involved in binding hGH to
the hPRLbp (Cunningham and Wells, 1991) while of these two only R178 is required for binding
to the hGHbp (Cunningham and Wells, 1989). These series of studies, on mPL-II, hGH, and
bPRL, demonstrates that one cannot generalize about features of a family of proteins with the
same biological activity from a study of one member.
None of the substitutions used in the loop region connecting putative helices 1 and 2 of the bPRL molecule led to a severe reduction in mitogenic activity (Figure 23), suggesting that these residues are not critically important for the interaction of the hormone with its receptor. Of the positions examined, replacement of residues H59 (with alanine and serine) and Q71, Q73, and Q74 (with valine) did result in a greater than 60% decrease in bioactivity; therefore, these amino acids may have a subsidiary role in the mitogenic activity of met-bPRL. Interestingly, the substitution of alanine for lysine at position 69 resulted in a more active met-bPRL molecule, suggesting that removal of the lysine side chain increases the ability of the hormone to interact with the receptor. Therefore, in bPRL this lysine residue could act to modulate receptor binding.

The sequences of the second half of the loop region joining putative helices 1 and 2 show strong homology across the growth hormone/prolactin/placental lactogen family (Figure 22; also see Goffin et al., 1992 for a more complete list of sequences in this region) and it has been suggested that this region may be important for the activity of all of the hormones in this family (Goffin et al., 1992). Residues in this region have been shown to be involved in the binding of hGH to the hGH and hPRL receptors (Cunningham and Wells, 1989, 1991) and in the binding of hPRL to the Nb2 PRL receptor as well as its mitogenic activity in the Nb2 cell assay (Goffin et al., 1992). The homology between bPRL and hPRL is particularly strong in this region: the only difference is position 64 (proline in bPRL, alanine in hPRL). However, in spite of the almost identical sequences in this region there are significant differences in the contribution of equivalent residues to the mitogenic activities on Nb2 cells of hPRL and bPRL (Table 5).

The role of individual amino acids in the 58 - 74 region of hPRL to the activity of this hormone was investigated in an alanine-mutagenesis study (Goffin et al., 1992) and while the bioactivities of the hPRL variants T60A, L63A, E67A were very similar to the corresponding met-bPRL variants, differences in activity were observed at the other positions (Table 5). Most notably, the hPRL variants S61A and Q74A showed increased levels of mitogenic activity (122% and 171%, respectively) while the activities of the met-bPRL variants (S61A and Q74V) were lower than that of unmodified met-bPRL (71% and 36%, respectively). A very striking difference between the two hormones was seen with the K69A variant: in hPRL this substitution
essentially abolished Nb2 cell bioactivity whereas in bPRL the mitogenic activity increased 1.5-fold. No bioactivity values were reported for hPRL variants carrying substitutions at positions Q71 or Q73.

Table 5. Comparison of the Effect of Single Amino Acid Substitutions in the Loop Region Joining Putative Helices 1 and 2 on the Bioactivity of bPRL, on the Bioactivity and Binding of hPRL to the Nb2 PRL Receptor, and on Binding of hGH to the hPRL and hGH Receptors

<table>
<thead>
<tr>
<th>Residuea</th>
<th>bPRL % Bioactivity</th>
<th>hPRLb % Bioactivity</th>
<th>Binding Abilityc</th>
<th>hGRc Binding Abilityf</th>
<th>hPRLbp</th>
<th>hGHbp</th>
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<td>58</td>
<td>2.4</td>
<td>1.4</td>
<td>4.4</td>
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<tr>
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<td>1.03</td>
<td>1.5</td>
<td>1.2</td>
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</tr>
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<td>S62</td>
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<td>77s</td>
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<td></td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
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<td>17</td>
</tr>
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<td>1.9</td>
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</tr>
<tr>
<td>Q74</td>
<td>Q69</td>
<td>36</td>
<td>171</td>
<td>0.76</td>
<td>0.7</td>
<td>0.91</td>
</tr>
</tbody>
</table>

a Position of corresponding residues in bPRL, hPRL, and hGH. Variants at these positions are all single alanine substitutions except residues 62, 68, and 70 of bPRL which were mutated to threonine, glycine, and asparagine, respectively.
b Data taken from Goffin et al., 1992.
c Data for all variants except P61A taken from Cunningham and Wells, 1991.
d Bioactivity refers to mitogenic activity in the Nb2 cell bioassay and is expressed as a percentage relative to unmodified bPRL or hPRL.
e Binding ability of hPRL to the Nb2 receptor is expressed as the ratio IC_{50} (mutant)/IC_{50} (hPRL). IC_{50} is the concentration at which each protein caused half-maximal displacement of \textsuperscript{125}I-labeled hPRL from the Nb2 receptor in Nb2 cell homogenates. Higher values indicate decreased ability to bind to the Nb2 receptor.
f Binding ability of hGH mutants for the extracellular part of the hPRLbp is expressed as K_d (mutant)/K_d (hGH). The dissociation constant K_d was determined for each mutant by competitive displacement of \textsuperscript{125}I-labeled hGH from the hPRLbp in the presence of 50 mM ZnCl\textsubscript{2}. Higher numbers indicate decreased ability to bind to the hPRLbp.
g Data for these variantshave been taken from Luck et al., 1989.
h Residue 64 is a proline in bPRL and an alanine in hPRL.
i The binding affinity of this variant is taken from Sakal et al., 1993.
Residue 64 is a proline in bPRL and an alanine in hPRL.
The binding affinity of this variant is taken from Sakal et al., 1993.

The homology between the sequences of bPRL and hGH in the second half of the loop region is not as strong as that between bPRL and hPRL. Although most of the differences are conservative (such as serine/threonine, isoleucine/leucine, arginine/lysine, and glutamate/glutamine), a number of the differences between the sequences of hGH and bPRL are marked, i.e., at positions 59, 61, 67, and 72 of bPRL (residues 54, 56, 62, and 67 of hGH). Interestingly, when compared across the hormone family the most divergent residues in this portion of the loop region are found in the first three of these positions. The binding affinity for the hPRL receptor of hGH variants containing alanine substitutions in the loop region has been examined (Cunningham and Wells, 1991; Sakal et al., 1993) (Table 5). Zn\(^{2+}\) was required for binding of all hGH variants to the hPRLbp, a situation unique amongst the hormone-receptor interactions discussed here. Human GH variants I58A, P61A, S62A, and N63A (corresponding to L63, P66, E67, and D68 of bPRL) each exhibited decreased binding to the lactogenic receptor. In addition, the hGH variant I58A had decreased binding affinity for the hGHbp. The other loop region amino acids which in hGH play a role in contacting the somatogenic receptor are F54, E56, R64, and Q68 (corresponding to H59, S61, K69, and Q73 of bPRL) (Cunningham and Wells, 1989).

Although in the present study the binding affinity of the met-bPRL variants to the Nb2 receptor was not determined, it was observed that none of the variants were able to inhibit the action of unmodified met-bPRL on the Nb2 cells (data not shown) indicating a possible correlation between low mitogenic activity and reduced receptor binding ability. Indeed, the hPRL variants least able to stimulate the growth of the Nb2 cells, namely P66A and K69A, were also the most affected in their ability to bind to the Nb2 PRL receptor while the variants S61A and Q74A showed an increase in both mitogenic activity and binding affinity (Table 5 and Goffin et al., 1992).

The differences between the contributions of equivalent residues within this highly conserved region of these related hormones again illustrates that although the same general region of each hormone may be involved in binding to the receptor molecules, the functional residues can be different in each case. Certain residues may be universally important:
abolished mitogenic activity in bPRL (Luck and Huyer, et al., 1992), hPRL (Goffin et al., 1992), and mPL-II (Davis and Linzer, 1989a). Substitution of this cysteine residue would result in disruption of the disulfide; thus, the importance of this amino acid to biological activity is most likely due to its role in stabilizing the conformation of the loop joining helices 1 and 2 with respect to the three-dimensional structure of the hormone. In addition, disruption of the disulfide bond likely would prevent the various residues, which together form the binding site, from attaining the conformation required for hormone-receptor binding. However, in contrast to the general importance of the cysteine residue to biological activity, the contribution of the other universally conserved residue, P66 (numbering according to bPRL), varies between hormones. For both hPRL and hGH this residue is important for activity: substitution of this amino acid with alanine decreased the mitogenic activity of hPRL by 4-fold and the binding to the Nb2 PRL receptor by more than 3-fold (Goffin et al., 1992) and decreased hGH binding to the hGHbp by 8-fold (Saka et al., 1993). However, the P66A variant of bPRL did not show a significant decrease in mitogenic activity (Figure 23). It is also very interesting that in spite of the almost total conservation of sequence in the loop region between hPRL and bPRL, the role of residues K69 and Q74 in the mitogenic activity of each hormone is very different: in hPRL K69A has almost no activity while in bPRL this variant has increased activity; and in hPRL Q74A has increased activity while in bPRL the mitogenic activity is reduced 3-fold. It is clear, therefore, that differences exist not only at the residue level between binding of PRLs and hGH to the lactogenic receptor, which might be anticipated because of the role of Zn$^{2+}$ in the activity of the latter, but also in the binding of different, but closely related, PRLs.

In general, the contributions of each of the loop region residues to the biological activities and/or receptor-binding ability of bPRL, hPRL, and hGH are quite different (Table 5). None of the residues are crucial to activity in all the hormones although replacement of H59 (F54 of hGH) does have a slightly deleterious effect on the mitogenic activity and/or binding ability of each of these hormones. Similarly, while there are loop region residues which are not involved in the activity of the individual hormones, there is no one residue in this region which is not involved in activity of any of these hormones except possibly T60 (S55 of hGH) which appears
to have a universally minor role. Some residues are required only for binding of a hormone to one of the receptors studied [e.g., E56 of hGH (for binding to the hGHbp) and S62 of hGH (for binding to the hPRLbp)] while others play roles in more than one set of activities [e.g., I58 of hGH (for binding to the hGH and hPRL receptors)] and P66 of hPRL/P61 of hGH (for binding to the Nb2 PRL receptor and hGHbp, respectively)]. These results illustrate that while the general region for receptor binding may be the same for members of the hormone family the identities of the specific residues involved are often quite different. These differences likely contribute to the specificity of the interactions between these related hormones and their receptors.

Of the many putative helix 4 residues examined, only replacements of R177 (discussed above), D178, and K181 (Figure 27) severely affected the mitogenic activity of bPRL. Substitution of the aspartate at position 178 with either serine or glutamate (similar to aspartate in terms of both size and charge) gave variants with bioactivities of 9%, suggesting that this residue is very important for the mitogenic activity of bPRL. The bioactivities of the variants K181A and K181L were both approximately 5% that of unmodified met-bPRL, and the bioactivity of K181R was 63%. Thus, while K181 appears to play a very important role in the mitogenic activity of bPRL, the requirement for a lysine at that position is not absolute since substitution with the functionally similar residue arginine gave rise to a variant with close to two-thirds the activity of the unmodified hormone. It is possible that the interaction between bPRL and the Nb2 PRL receptor is enhanced by a charge-charge interaction involving the lysine at position 181. Alternatively, the positive charge on the lysine could be required to stabilize the bPRL structure via a hydrogen bond: the corresponding residue in hGH, K172, is important for the interactions between hGH and the hGH and hPRL receptors, but only the aliphatic portion of the side chain contacts the receptor molecules, the charged portion is involved in forming a hydrogen bond to D169 in the core of the hormone (Cunningham and Wells, 1993; de Vos et al., 1992). In this regard, it may be noted that D178 of bPRL corresponds to D169 of hGH; therefore, it is possible that the role of D178 in the activity of bPRL may be a structural one rather than one of directly contacting the receptor.
Table 6. Comparison of the Effect of Single Amino Acid Substitutions in Putative Helix 4 on the Bioactivity of bPRL and on the Binding of hGH to the hPRL and hGH Receptors

<table>
<thead>
<tr>
<th>Residue&lt;sup&gt;b&lt;/sup&gt;</th>
<th>bPRL</th>
<th>hGH</th>
<th>% Bioactivity&lt;sup&gt;d&lt;/sup&gt;</th>
<th>hPRLbp</th>
<th>hGHbp</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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<td></td>
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<tr>
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<td>Y164</td>
<td>58</td>
<td>2.1</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>L175</td>
<td>F166&lt;sup&gt;e&lt;/sup&gt;</td>
<td>58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R176</td>
<td>R167</td>
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<td>770</td>
<td>0.75</td>
<td></td>
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<tr>
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</tr>
<tr>
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<td>D169&lt;sup&gt;f&lt;/sup&gt;</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
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<tr>
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<td>D171</td>
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<td>7.1</td>
<td></td>
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<td>14</td>
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</tr>
<tr>
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<td>F176</td>
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<tr>
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<tr>
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<td>4.5</td>
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</tbody>
</table>

<sup>a</sup> Data are taken from Cunningham and Wells, 1991.

<sup>b</sup> Identity and position of corresponding residues in bPRL and hGH. All were replaced with alanine unless otherwise indicated.

<sup>c</sup> Binding ability of hGH to the extracellular portions of the hPRLbp and hGHbp is expressed as $K_d$ (mutant)/$K_d$(hGH). The dissociation constant $K_d$ was determined for each mutant by competitive displacement of $^{125}$I-labeled hGH from the hPRLbp (in the presence of 50 μM ZnCl$_2$) or the hGHbp.

<sup>d</sup> % bioactivity is the mitogenic activity in the Nb2 cell bioassay and is expressed relative to wild-type bPRL (100%).

<sup>e</sup> No replacements of G161, L162, and F166 of hGH were reported.

<sup>f</sup> Not enough of D169A and M170A of hGH were produced in E. coli for analysis of receptor-binding abilities.

<sup>g</sup> D178 of bPRL was replaced with glutamate (D178E).

<sup>h</sup> T175 of hGH was replaced with serine (T175S).
Alanine-scanning mutagenesis of hGH revealed that helix 4 plays a very important role in the interactions between this hormone and the human growth hormone and prolactin receptors (Cunningham and Wells, 1989, 1991). However, inasmuch as detailed studies of the contribution of this region to the biological activities of related hormones has not yet been carried out, the general importance of this region of helix 4 in hormone-receptor interactions has not been established. In hGH helix 4 amino acids are involved in binding to both the hGHbp and the hPRLbp, and although the binding sites are not identical they do overlap. A comparison between the contribution of helix 4 residues to the activities of hGH and bPRL is given in Table 6. As was noted for the residues in the loop region joining helices 1 and 2, the residues in helix 4 have a variety of roles in binding to and activating the different receptor molecules.

Thus, in hGH, residues K172, F176, and R178 interact with both the hGH and hPRL receptors; D171 and V185 are only involved in binding to the hGHbp; and R167, K168, and E174 are only required for binding to the hPRLbp (Cunningham and Wells, 1991). The very large reduction in the binding ability of hGH to the hPRLbp for the variant E174A is a result of disruption of the Zn$^{2+}$-binding site. The corresponding residues in bPRL and hGH which have important roles in the activities of both these hormone are R176, R177 and K181 of bPRL (R167, K168 and K172 of hGH): the hGH variants R167A, K168A and K172A exhibited reduced affinity for the hPRLbp but not for the hGHbp. Marked differences between the hormones are seen at positions T184, Y185, K187, and R192 of bPRL: replacements of the corresponding hGH residues (T175, F176, R178, and R183) disrupted binding to both the hGH and hPRL receptors while replacements of the bPRL had little or no effect on the ability of the hormone to activate the Nb2 PRL receptor. Thus, while there appear to be some similarities between the binding sites of hGH for the hPRLbp and of bPRL for the Nb2 PRL receptor there are also some significant differences.
Figure 28. Ribbon representation of the putative structure of bPRL indicating the binding site for activation of the Nb2 PRL receptor. The model does not contain the first 14 N-terminal amino acids or the C4 - C11 disulfide. The truncated N-terminus is located in the upper right-hand corner and the C-terminus, joined in a disulfide bond (coloured greenish-yellow) to a cysteine near the C-terminus (C191 - C199) is positioned just below the N-terminus. The side chains indicated are those which in the present study were identified as being involved in the mitogenic activity of bPRL. Substitutions of these amino acids, primarily with alanine, resulted in a reduction in mitogenic activity. The disulfide bond joining the loop region between helices 1 and 2 to the cysteine within helix 4 is also shown (C58 - C174) (coloured greenish-yellow) and it located in the middle bottom of the representation of the hormone structure.
4.3 Residues Responsible for the Bioactivity of Met-bPRL in the Nb2 Assay

Systematic replacement of residues within the loop region joining putative helices 1 and 2 and within the C-terminal two thirds of helix 4 has identified a number of residues which play a role in the mitogenic activity of bPRL. These include H59, Q71, Q73, Q74, L171, R176, R177, D178, K181, and D183. When mapped onto a putative structural model of bPRL, obtained by molecular modeling of bPRL from the structure of hGH, these residues form a patch covering the middle of helix 4 and the latter third of the loop region (Figure 28). The general location of these amino acids is very similar to that identified as being involved in the interaction between hGH and the hPRLbp (Figure 29 and Cunningham and Wells, 1991). Indeed, as discussed above, the similarity between the binding sites extends beyond general location to certain specific residues: most notably, the side chains of the equivalent residue pairs R177 and K181 of bPRL and K168 and K172 of hGH are similarly positioned for interacting with the receptors.

The putative molecular model of bPRL can be used to shed light on the contribution of particular amino acids to the mitogenic activity of the hormone. Replacements of any of residues H59, L171, and D178 resulted in a reduction in mitogenic activity; however, the side chains of L171 and D178 are on the interior-facing sides of helices (Figure 30). The internal location of the side chains of L171 and D178 was predicted by the helical wheel diagram (Figure 24). Therefore, the role of these residues is most likely a structural one rather than one of receptor binding. Replacements of these residues may distort the protein structure and indirectly cause reductions in mitogenic activity. Interestingly, L171 and D178 correspond to the hGH residues L162 and D169 which are found in hydrophobic clusters and therefore likely have structural roles: L162 is part of a group of hydrophobic residues from the region between helices 2 and 3, and from helices 1 and 4; while D169 is part of the core of the four-helix bundle (de Vos et al., 1992). Other amino acids within the binding site which may also support the general structure are L63, P64, and P66 in the loop region joining helices 1 and 2. L63 corresponds to I58 of hGH, and although alanine scanning mutagenesis identified this hGH residue as being critical for interaction with both the hGHbp and the hPRLbp (Cunningham and Wells, 1991), the crystal structure of the hormone-receptor complex revealed that I58 is part of a hydrophobic cluster and
does not directly contact the receptor molecule (de Vos et al., 1992). L63 is, like I58, a hydrophobic residue and in the putative structural model of bPRL its side chain points into the hormone molecule. The side chains of P64 and P66 are similarly positioned in that they also face into the interior of the protein (Figure 30). However, since replacing L63 or either of the proline residues with alanine did not cause a major reduction in mitogenic activity (51%, 61%, and 70%, respectively), individually the contributions of these amino acids to the maintenance of the structure of the binding site does not appear to be very great.

The functional roles of K69, Q71, Q73, and Q74 can also be inferred from the putative model of bPRL. The side chains of all of these amino acids appear to be very solvent-accessible and are therefore well positioned for interacting with a receptor molecule and appear less likely to have structural roles (Figure 28). The mitogenic activity of variants in which any of these three glutamine residues was replaced by valine or alanine was approximately 3-fold less than that of unmodified met-bPRL (Figure 23 and Table 5). It is possible that the space vacated by the replaced glutamate could be filled by one of the two glutamate residues remaining and that this movement of side chains might mitigate the effect of replacement of any one of the glutamates in this region. Therefore, while the observed reduction in bioactivity from the individual replacement of these residues is not particularly large it does raise the possibility that these amino acids may play a role in interacting with the Nb2 PRL receptor. On the other hand, replacement of the lysine at position 69 with an alanine resulted in an increase in mitogenic activity, to 1.5-fold greater than that of unmodified met-bPRL (Figure 23 and Table 5). This lysine residue may, by virtue of its large side chain, physically hinder slightly the interaction between bPRL and the Nb2 PRL receptor: contact between these proteins may be improved by the replacement of K69 with an amino acid that has a less bulky side chain. Alternatively, it is possible that there may be a repulsion effect between bPRL and the Nb2 PRL receptor as a result of the positive charge on the lysine residue. In this case, replacement of the lysine with an aspartate or glutamate may result in a variant with even greater activity.
Figure 29. Ribbon representation of the structure of hGH indicating residues within the site 1 and site 2 binding regions for the hPRLbp. The N-terminus is located in the upper right-hand corner. A portion of the C-terminus could not be modelled; therefore the structure shown lacks the terminal 4 amino acids and the C182 - C189 disulfide. The truncated C-terminus is located just below the N-terminus in the middle of the right-hand side of the figure. The residues indicated are those sites where alanine substitutions resulted in significant reductions in site 1 binding affinity to the hPRLbp (H18, H21, F25, I58, S62, N63, R167, K168, K172, E174, T175, and R178). The role of H18 and E174 is coordinating the Zn$^{2+}$ molecule required for binding hGH to the hPRLbp. Alanine substitutions of D116 and E119 decreased receptor binding at site 2. Also indicated are residues within the site 1 binding region for the hPRLbp which are involved in binding hGH to the hGHbp (E56, L58, R64, K172, F176, R178). The complete set of alanine mutants that reduced binding to the hGHbp, comprising residues outside the binding site for the hPRLbp, is not shown. The disulfide joining the loop region between helices 1 and 2 to helix 4 (C53 - C165) is indicated as a yellow tube.
Figure 30. Ribbon representation of the putative structure of bPRL indicating residues within the binding site for the Nb2 PRL receptor. The truncated N-terminus lacking the C4 - C11 disulfide is in the upper right-hand corner and the C-terminus, part of the C191 - C199 disulfide (coloured yellow) is located just below the N-terminus in the middle of the right-hand side of the figure. The C58 - C174 disulfide is indicated as a shadowed greenish-yellow tube in the bottom middle of the figure. Residues indicated include those which are involved in activating the Nb2 PRL receptor (K69, Q71, Q73, Q74, R176, R177, K181, and D183), those whose replacement decreased the mitogenic activity of bPRL but which most likely play a structural role (H59, L171, and D178) and those whose replacement had little effect on activity (R21, L63, P64, P66, S180, and K187). Residues R176, D178, and D183 are not labeled.
The hGH residues which correspond in general location to K69, Q71, Q73, and Q74 of bPRL are N63, R64, E65, and E66 (Figures 28 and 29). Replacements of these residues altered binding to the hPRLbp and/or to the hGHbp (Cunningham and Wells, 1991; Table 5). The role of R64, E65, and E66 is likely related to the charged nature of their side chains: arginine is positively charged and glutamate is negatively charged. The E65A hGH variant exhibited enhanced binding affinity for the hGHbp and a nearly two-fold increase in on-rate of the hormone to the receptor (Cunningham and Wells, 1993) but decreased affinity for the hPRLbp. In contrast, the E66A hGH variant exhibited decreased affinity for the hGHbp with no change in the affinity for the hPRLbp (Cunningham and Wells, 1991). These electrostatic interactions as well as that involving the positive charge on the arginine at position 64 are believed to be important for modulating the association of hGH with the receptor molecules (Cunningham and Wells, 1993). These results suggest that the positive charge on K69 in bPRL, rather than the size of its side chain, is likely important in the interaction between the hormone and the Nb2 PRL receptor.

The putative position of residues R21 and K187 in bPRL, of which the corresponding residues in mPL-II (namely R14 and K179) play important roles in mitogenic activity, are on the edge of the patch of amino acids in helix 4 and the loop region which together seem to form the binding determinant for the Nb2 PRL receptor (Figure 30). The side chains of both these amino acids appear to be located on the exterior of the bPRL molecule and are therefore in a favourable position for interacting with the receptor. Non-conservative substitutions of both R21 and K187 resulted in minor reductions in mitogenic activity, with the effect of the R21 substitutions being slightly greater (Table 4). Consideration of the putative location of these residues along with the apparently minor roles that they play in the mitogenic activity of bPRL suggests that they are located on the edge of the binding site for the Nb2 PRL receptor. Thus, although there is a great deal of sequence homology between bPRL and mPL-II within the portion of putative helix 4, which in bPRL contains the binding determinants for the Nb2 PRL receptor (Figure 22), the binding sites for these related hormones to the same receptor molecule are obviously slightly different.
The requirement for Zn\textsuperscript{2+} in the formation of the hGH-hPRLbp complex provides a further complexity to an analysis of lactogen-PRLbp interaction. Zn\textsuperscript{2+} is required for binding of hGH to the hPRLbp but not for the interaction of either hGH with the hGHbp or hPRL with the hPRLbp (Cunningham et al., 1990a). The hGH ligands which coordinate the Zn\textsuperscript{2+} are H18 and E174. In addition, although H21 does not bind the metal ion it is required to correctly orient the side chain of E174 (Somers et al., 1994). Throughout the family of hormones all three of these residues are highly conserved, particularly the two histidines (Figure 22). However, although in bPRL the residue equivalent to E174 of hGH (namely D183) does appear to play a role in the mitogenic activity, neither of the histidine residues is important and Zn\textsuperscript{2+} does not appear to be required for binding bPRL to the Nb2 PRL receptor (Luck et al., 1990; Dr. Luck, Oberlin College, Oberlin, OH, personal communication). Indeed, the only members of the hormone family for which Zn\textsuperscript{2+}-mediated binding to a receptor been reported are hGH and hPL: both require Zn\textsuperscript{2+} to bind the hPRLbp (Lowman et al., 1991). Therefore, the role played by D183 of bPRL in activating the Nb2 PRL receptor is quite different from the role of the equivalent hGH residue, E174.

4.4 Receptor Dimerization

Human GH contains two binding sites for the hGHbp: the hormone first binds a receptor molecule at site 1 and then a second hGHbp is bound at site 2 (Figure 31) (Cunningham et al., 1991; de Vos et al., 1992). The residues comprising site 1 form a patch covering sections of helices 1 and 4 and part of the loop region joining helices 1 and 2, while site 2 residues are located at the N-terminus of the hGH molecule and in the middle of the third helix. Receptor oligomerization is a common mechanism for signal transduction (Ullrich and Schlessinger, 1990) but the interaction between hGH and the hGHbp is unique in exhibiting a stoichiometry of one hormone to two receptors. There is a great deal of homology between members of the growth hormone family and it is likely that this extends to a common mechanism of receptor binding. Indeed, there is evidence indicating that binding of PRL to the PRLbp also occurs through a two-site mechanism (Elberg et al., 1990; Gertler et al., 1993; Goffin et al., 1994; Shiu et al., 1983) although definitive proof of whether this indeed occurs has not yet been obtained.
Evidence that bPRL contains a binding site analogous to site 2 of hGH was obtained from an examination of the glycine at position 129. The G129R variant of bPRL was found to have absolutely no mitogenic activity in the Nb2 lymphoma cell system. This result strongly suggests that the introduction of a charged, bulky side chain at position 129 blocks the formation of a productive interaction between bPRL and the Nb2 PRL receptor. The corresponding residue in hGH is G120, and substitution of this amino acid with arginine, with its charged, bulky side chain, resulted in a mutant which was able to bind the hGHbp at site 1 but which blocked binding at site 2 (Fuh et al., 1992). This variant was a potent antagonist of hGH-induced cell proliferation: binding of the mutant prevented receptor dimerization and the concomitant mechanisms of signal transduction. Similarly, replacement of G129 of hPRL (equivalent to G120 of hGH) reduced the binding affinity and the Nb2 cell bioactivity by two to three orders of
magnitude (Goffin et al., 1994) In addition, transgenic mice expressing bGH or hGH analogs with mutations in the third α-helix, including residue G120, exhibited a dwarf phenotype (Chen et al., 1991a, 1994, 1995). Based on the homology which exists between the binding of hGH to the hGH and hPRL receptors, it is likely that the effect of the G129R mutation in bPRL results from interference with site 2 binding. A model of bPRL indicating the putative locations of site 1 and site 2 is shown in Figure 32.

The site 2 residues, as identified in hGH and bGH, are predicted to form a cleft in the third α-helix with the glycine (position 120 in hGH and 119 in bGH) located at the centre and flanked by larger residues such as aspartate and leucine (D116 and L124 of hGH, and D115 and L123 of bGH) which may interact with the receptor molecules (Chen et al., 1995). That a small residue is satisfactory for function was confirmed by the creation of transgenic mice expressing a G120A variant of hGH: these mice did not exhibit a growth-suppressed phenotype. Therefore, the activity of the hormone likely depends on the presence of a small amino acid at that locus and not on the glycine per se (Chen et al., 1994). The severe effects arising from either replacement of the glycine with arginine (Chen et al., 1994, 1995; Fuh et al., 1992; Goffin et al., 1994; this study) or its deletion (Chen et al., 1995) further suggests that the primary structure of the third α-helix is critical for hormone activity.
Figure 32. Ribbon representation of the putative structure of bPRL indicating residues within the putative site 1 and site 2 binding sites. The truncated N-terminus lacking the C4 - C11 disulfide is located in the upper right-hand corner and the C-terminus, which forms part of the C191 - C199 disulfide (indicated in greenish-yellow), is positioned just below the N-terminus. The C58 - C174 disulfide is shown as a shadowed, greenish-yellow tube. The residues indicated are those which when substituted with other amino acids, primarily alanine, resulted in a variant with reduced mitogenic activity. These residues may therefore form the binding determinants for site 1 and site 2 binding to the receptor. Residues K69, Q71, Q73, and Q74 are on the loop region joining helices 1 and 2, residues R125, E128, and G129 are on helix 3, and residues R176, R177, D178, K181, and D183 are on helix 4.
4.5 Species Specificity of the Hormone Binding Site

While not a common characteristic of members of the hormone family, the ability to bind with high affinity to different types of receptors is not limited to hGH. Two species of placental lactogen from the mouse (PL-I and PL-II) are capable, along with mPRL, of binding to the mouse prolactin receptor (Davis and Linzer, 1989b), and ovine placental lactogen (oPL) is one of the few hormones other than hGH that binds with high affinity to the hGHbp (Carr and Friesen, 1976). The overall sequence identity between oPL and hGH is small (26%) and although the degree of homology is greater amongst the residues which in hGH contact the hGHbp (Colosi et al., 1989) there are enough differences to suggest that oPL and hGH have overlapping but different sets of important binding residues. One dissimilarity is the residue occupying the position equivalent to D171 of hGH: this amino acid is a serine in oPL. This substitution is particularly interesting since it relates to recent evidence suggesting that the identity of the amino acid at this position is a major determinant of whether a growth hormone from one species will bind to a receptor from another species, a phenomenon called species specificity (Souza et al., 1995). While the growth hormone receptors from many species bind hGH as well as their own growth hormones, the hGHbp will bind only growth hormones from primates. It was suggested that the basis for this specificity is the arginine at position 43 in the hGHbp. R43 contacts both D171 and T175 of hGH and while the threonine is conserved, the aspartate is not. In all non-primate growth hormones, in many prolactins, and in PL-II from rat and mouse a histidine residue is found at the position corresponding to 171 of hGH while a few hormones including oPL, oPRL, and bPRL display a serine at this locus. Neither bPRL nor oPRL bind to the hGHbp (Carr and Friesen, 1976); therefore, the major determinant of species specificity is more likely to be the incompatibility of a histidine at position 171 with R43 of the receptor than an absolute requirement for aspartate or serine at that locus. With respect to bPRL, the putative model suggests that the serine at this position (S180) is located within the patch of amino acids that has been identified as being important for the mitogenic activity of the hormone. In addition, its side chain appears to be well positioned for interacting with the receptor (Figure 30). But since replacement of this serine with alanine (variant S180A) did not cause a large reduction in
replacement of this serine with alanine (variant S180A) did not cause a large reduction in mitogenic activity (Figure 27 and Table 6), the serine per se cannot be essential for activity. However, in light of the above evidence that this locus may play a role in species specificity, it would be very interesting to examine the effect that replacement of S180 with a bulky residue such as histidine might have on the mitogenic activity of bPRL.

4.6 Conclusions and Future Directions

Regulation of the many biological processes in which prolactin, along with the other members of the hematopoietic family of hormones, is involved is accomplished through the interaction of the hormone with receptor molecules. Receptor binding initiates the signal transduction mechanisms which ultimately give rise to the various biological effects. Therefore, elucidation of the specific contacts involved in the molecular interaction between each hormone and its receptor is an essential component of the body of knowledge required for each hormone in order to fully exploit their commercial and medical potentials, particularly as related to the treatment of various diseases.

The current structure-function investigation into the interaction of bovine prolactin with the Nb2 PRL receptor has confirmed the generality of the binding site and highlighted the differences that exist at the residue level with respect to other related hormones. As predicted from the sequence homology between members of the family of growth hormones, prolactins, and placental lactogens, the binding site of bPRL for the Nb2 PRL receptor appears to be very similar to that reported for binding of hGH to both the hGH and hPRL binding proteins. The data show that bPRL residues within the C-terminal half of the loop region joining putative helices 1 and 2 as well as within the central portion of putative helix 4 are involved in promoting the mitogenic activity of the hormone. Amino acids R177 and K181 were demonstrated to be critically important for activity of bPRL while replacements of other residues, including Q71, Q73, Q74, R176, and D183, indicated that they may also play a role in contacting the receptor, or may, in the cases of L171 and D178, play a structural role in maintaining the global structure of the binding site.
However, while the general location of these residues is similar to that of the residues which in related hormones are involved in contacting receptor molecules, the identity among hormones does not extend to the level of specific amino acids. For example, residues R21 and K187 were found not to be very important for the mitogenic activity of bPRL, while the corresponding mPL-II residues were shown to be very important for the mitogenic activity of that hormone (Davis and Linzer, 1989b). Differences between the residues involved in receptor binding exist between bPRL and all the other hormones for which structure-function analyses have been performed and are particularly striking between bPRL and hPRL. While the sequence homology between these hormones within the binding epitope region is very high, there are significant differences in the contribution that some of the corresponding residues in bPRL and hPRL make to the mitogenic activity. For example, P61A of hPRL exhibited a four-fold reduction in mitogenic activity (Goffin et al., 1992) while the activity of P61A of bPRL was only slightly reduced. In addition, the K69A variant of hPRL had very low bioactivity and was severely affected in its ability to bind to the Nb2 PRL receptor (Goffin et al., 1992) but the K69A variant of bPRL exhibited increased mitogenic activity. The differences between the interaction of bPRL with the Nb2 PRL receptor and the interactions of hGH with the hGH and hPRL receptors are also quite marked. One major difference between the interactions of these two hormones with a lactogenic receptor is that Zn$^{2+}$ is required for the binding of hGH to the hPRLbp (Cunningham and Wells, 1991) but not for bPRL binding to the Nb2 PRL receptor. Thus, although the corresponding residues D183 of bPRL and E174 of hGH are both important for the interaction of these hormones with a lactogenic receptor, the roles of these residues are not identical since E174 of hGH is involved in coordinating the Zn$^{2+}$ ligand. Another marked difference between the two hormones is seen with the contribution of the corresponding residues E67 of bPRL and S62 of hGH: the mitogenic activity of the E67A met-bPRL variant was almost identical to that of unmodified met-bPRL while the ability of the S62A variant of hGH to bind to the hPRLbp was severely reduced (Cunningham and Wells, 1991).

The comparison of sequences and analysis of residues conserved between the related growth hormones, prolactins, and placental lactogens has not, in general, been very informative.
for identifying residues which form the receptor binding sites. As noted repeatedly above, residues which are highly conserved throughout the family of hormones do not play equally important roles in interacting with the receptor molecules. However, as shown from a comparison of the hormones hPL, pGH, and hPRL with hGH, consideration of sequence divergence as a basis for predicting which residues form binding sites can be misleading. In these examples the sequence variations within the binding site are the same as that over the entire hormone molecule (Cunningham and Wells, 1989). Thus, a number of sequence differences throughout the disparate regions that form the binding sites give rise to the functional differences between the hormones.

The many differences between the residues involved in the interactions among these related hormones and their receptors in spite of the similarities in the general locations of the binding sites likely contribute to hormone-receptor binding specificity. Although the receptors for this family of hormones also belong to a family of proteins (Idzerda et al., 1990) they are quite different in terms of amino acid sequence. Therefore, the portions of these receptors with which the hormones interact contain a variety of residues and it is impossible that a single set of hormone residues could bind to each receptor. In addition, although the members of the growth hormone/prolactin/placental lactogen family of hormones are evolutionarily related (Miller and Eberhardt, 1983) these hormones have continued to evolve separately from each other and the different roles that individual residues have in activating receptors may be a consequence of this evolutionary divergence.

In this study evidence was also obtained which suggests that bPRL, like hGH, contains two separate receptor binding sites and that signal transduction therefore follows receptor dimerization. Replacement of the glycine at position 120 with arginine totally abolished the mitogenic activity of bPRL: this effect may be the result of the arginine residue hindering contact between the hormone and the receptor at site 2 either by virtue of its size or of its charge. The mechanism of the interaction between hGH and the hGHbp or the hPRLbp is one of sequential dimerization wherein the affinity of site 1 for the receptor is higher than that of site 2 (Fuh et al., 1992, 1993). However, a study of the interaction between hPRL and the Nb2 lactogenic receptor
suggested that this mechanism may not be universal amongst the family of hormones: the binding affinity of both hPRL sites for the Nb2 PRL receptor appeared to be similar (Goffin et al., 1994). Further study is needed to determine which mechanism is followed with respect to bPRL binding to the Nb2 PRL receptor.

Significant progress has been made towards obtaining an understanding of the structure-function relationship between bPRL and the Nb2 PRL receptor but much work remains to be done. The binding site of hGH for the hGHbp and the hPRLbp includes residues within helix 1 as well as within the loop region and helix 4 (Cunningham and Wells, 1991), and amino acids within the N-terminal region of rat PRL have been shown to be involved in the mitogenic activity of this hormone (Maruyama et al., 1994). Therefore, the contribution of putative helix 1 residues to the mitogenic activity of bPRL should be determined, both by alanine-scanning mutagenesis and by replacement of residues believed to be within the binding site by amino acids which might have a more disruptive effect on receptor binding by virtue of their size or charge. In this regard it is of interest that alanine-scanning mutagenesis of putative site 2 residues of hPRL did not identify side chains involved in activating the Nb2 PRL receptor, but replacement of certain of these residues with tryptophan or arginine did provide evidence for a second binding site (Goffin et al., 1994). The data obtained regarding the effect of the G129R mutation provides a tantalizing glimpse into the mechanism of receptor binding; i.e., that bPRL contains two receptor binding sites: additional mutations should be created in this region in order to further explore this possibility. It may even be possible to generate variants which, by acting as antagonists to PRL, could be useful in the treatment of disorders such as hyperprolactinemia.

While a structure-function analysis provides a great deal of information with respect to the contribution of specific residues to the functional activity of a protein, a crystal structure is necessary to determine whether residues which appear to be functionally important actually play a structural role in maintaining the binding site rather than directly interacting with the receptor. In addition, the validity of the model of receptor activation could be tested by the creation of specific mutants that, as suggested by molecular modeling based on the coordinates of the wild-type structure, would be expected to severely interfere with the hormone-receptor interaction.
However, a relatively large amount of pure protein is required in order to perform a structural analysis of prolactin. Unfortunately, the system used in this study for the isolation of active met-bPRL is not amenable to large-scale production of protein. There are many problems associated with efficient production of recombinant proteins, not the least of which is producing a correctly folded and soluble protein. Although there are a wide variety of *E. coli* expression systems available for high-level expression of eukaryotic proteins in *E. coli*, these often lead to accumulation of the foreign protein in insoluble inclusion bodies. As it is possible that specific mutations generated in the course of a structure-function analysis could affect the ability of the recombinant protein to refold correctly, and thereby interfere with an evaluation of the contribution of the residue in question to the biological activity of the protein, it is very important to assess the conformation of the mutants. While production of recombinant proteins in a soluble form does not guarantee that mutations will not cause any structural modification of the protein, it does eliminate the problems associated with solubilization and refolding of recombinant proteins from inclusion bodies. Thus, an investigation into alternative methods of producing the hormone, particularly those involving eukaryotic or insect cell cultures, may prove fruitful in terms of generating larger amounts of soluble, active bPRL than could be obtained with transformed cultures of *E. coli*.

The present work provides a significant contribution to the sum of knowledge about the interaction between bPRL and the Nb2 PRL receptor, and, as a consequence of the homology between prolactin and other hormones, lends support to the suggestion that there is a common mechanism of receptor binding and activation within the hematopoietic hormone family. Further study into the interaction between bPRL and the Nb2 PRL receptor will allow identification of all the bPRL residues that play a role in activating this receptor and will also, by virtue of the differences that exist between this hormone-receptor pair and others that have been examined, lead to greater understanding of the general mechanism of receptor binding by members of the growth hormone/prolactin/placental lactogen hormone family.
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