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IN VIVO EFFECT OF GROWTH HORMONE ON DNA SYNTHESIS AND EXPRESSION OF MILK PROTEIN GENES IN THE RABBIT MAMMARY GLAND

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The aim of tis work was to show whether growth hormone (GH) is able to directly induce growth and functional differentiation of the mammary gland. We have shown that i.m. injections of prolactin and to lesser extent injections of growth hormone increased DNA synthesis in the mammary gland of pregnant rabbits. Injections of pituitary and recombinant bovine growth hormone (GH), similarly to prolactin, could also induce the expression of milk protein genes — caseins αS1 and β and whey acidic protein (WAP). However, in contrast to prolactin, growth hormone failed to induce the synthesis of casein proteins. Lactogenic hormones act through binding to receptors in target tissues. Prolactin receptors were shown to be abundant in the rabbit mammary glands but no specific binding sites for ^{125}I-labelled GH have been found in membranes isolated from mammary glands of pregnant or lactating rabbits. The specificity of hormone binding was examined using unlabelled hormones as competitive inhibitors of ^{125}I-labelled prolactin. Bovine and recombinant bovine growth hormone did not displace prolactin from its receptors, thus excluding the possibility of action of GH through lactogenic receptors. Our results support the hypothesis that GH may act directly on the mammary gland and independantly from prolactin; however, the mechanism of its action is still unknown.

Key words: *mammary gland, DNA synthesis, milk protein genes, expression, prolactin, growth hormone*

INTRODUCTION

Growth and differentiation of mammary epithelial cells are regulated by hormones and growth factors and by the interactions of cells with their extracellular tissue environment such as extracellular matrix (1, 2). Prolactin, the main lactogenic hormone, can induce expression of milk protein genes both *in vivo* and *in vitro*. In the cultures of primary mammary epithelial cells and in mammary gland explants, prolactin in the presence of insulin and hydrocortisone maximally induces accumulation of milk protein mRNAs (3).

Growth hormone (GH) is a member of a family of hormones that also includes prolactin and placental lactogens. There is evidence that growth hormone has lactogenic activity in the *in vivo* experiments on mammary glands. Exogenous administration of bovine GH — natural or recombinant — increases milk production in lactating dairy cows (4). It is not clear whether action of growth hormone results from direct stimulation of the mammary gland to produce milk components or from increasing utilization of nutrients for the synthesis of milk. In most *in vitro* experiments only human growth hormone exhibits lactogenic activity. Human GH binds specifically to membrane-bound and solubilized prolactin receptors from bovine mammary gland (5). Growth hormones derived from other mammalian species do not directly affect mammary function (5—7). However, recently evidence has been presented that bovine growth hormone (bGH) has a direct effect on mammary cells and on the expression of milk protein genes. In the organ cultures of virgin mice (8) or pregnant rabbit (9) mammary glands, bGH at a concentration of 1 μg/ml induced expression of milk protein mRNAs.

The mechanism of action of growth hormone on the mammary gland at the molecular level is unknown. Lactogenic hormones like prolactin or human growth hormone act through binding to prolactin receptors in the target tissue. Prolactin receptor becomes abundant in the rabbit mammary gland as pregnancy proceeds and it is at its higest level in the lactating animal (10). GH receptors in mammary tissue have not yet been identified using conventional binding assays (11, 12). However, GH receptors were identified in the rat mammary gland by immunocytochemistry (13) and GH receptor mRNA has been found in mammary glands of cows (14), rabbits (15), rats (16) and human (17).

The objective of this study was to investigate whether growth hormone can induce expression of milk protein genes *in vivo* in the rabbit mammary gland, independently from prolactin. In addition, some aspects of the mechanism of action of growth hormone on the mammary gland have been studied.

MATERIALS AND METHODS

Animals and tissues

New Zealand female rabbits were used in their first pregnancy or lactation. Animals were killed by cervical dislocation (in the local abbatoire) at day 15 or 25 of pregnancy or at day 5 of lactation, their mammary glands were excised, cleared from most adjacent muscule, fat and connective tissues, mammary fragments (explants) were prepared and the rest of the gland was frozen at −80°C for further analysis.

Hormone injections

Bovine prolactin (IFZŻ, Jabłonna, Poland), bovine growth hormone (Calbiochem) or recombinant bovine growth hormone (Monsanto) were injected intramusculary in 4 mg doses three

times into rabbit femals between days 13—15 of pregnancy at 12-hour intervals according to procedure described by Houdebine (18) and Zwierzchowski *et al.* (19). Control rabbits were injected with physiological salt solution (PBS). Twelve hours after the last injection the animals were killed and mammary glands were excised. Three animals were used in each experimental group.

Measurement of DNA synthesis

Fragments of mammary glands taken from pregnant rabbits previously treated with hormones were cut into explants and incubated for 4 h at 37°C in Medium 199 (Gibco) containing 1 μCi/ml [^{3}H] thymidine (Amersham, sp. act., 22 Ci/mmol). DNA synthesis was estimated as described by Jahn *et al.* (20).

Measurement of casein synthesis

Explants were incubated for 4 h at 37°C in Medium 199 (Gibco BRL) containing 1 μCi/ml [^{3}H] leucine (70 Ci/mmol) and casein synthesis was measured by the calcium-rennin precipitation method described by Juergens *et al.* (21). Alternatively, explants were incubated under the same conditions as described above in the medium containing 20 μg/ml L-[^{35}S] methionine (NEN, 1000 Ci/mmol). Then, caseins were precipitated by rennin and calcium, dissolved in 1 × SDS gel-loading buffer (22) and separated on 15% polyacrylamide gels in buffer containig 25 mM Tris pH 8.3; 250 mM glycine, 0.1% SDS. Electrophoretically separated caseins were transferred from the gels to nitrocellulose filtres (BA85, Schleicher and Schuell) using electrobloting and the filters were autoradiographed at −80°C in ligtht-proof casettes with Hyperfilm (Amersham).

Northern blot analysis

Accumulation of β-, αS1-casein and whey acidic protein (WAP) mRNAs was estimated in mammary gland tissues prepared from pregnant rabbits which had been previously injected with hormones. Total RNA was extracted by guanidinum thiocyanate phenol-chloroform method (23). Ten μg of RNA was electrophoresed on 1.5% agarose (Gibco-BRL), transferred to Hybond-N nylon membrans (Amersham) and hybridized for 16—20 hours to rabbit β-casein, αS1-casein or WAP cDNA probes (a gift of Dr L. M. Houdebine, INRA, Jouy-en-Josas, France) labelled with [α-^{32}P] dCTP. After hybridization filters were washed with 2 × SSPE, 0.1% SDS at room temperature and then with 1 × SSPE and 0.1 × SSPE, 0.1% SDS at 65°C according to the manufacturer's instructions. Filters were autoradiographed at −80°C in light- proof casettes with Hyperfilm (Amersham) placed between two amplifying screens (Dupont, Cronex).

Measuring of prolactin and GH receptors

Preparation of ^{125}I-labelled prolactin and growth hormone

Radioiodinated prolactin and growth hormone were prepared by a modification of the chloramine-T method described by Greenwood *et al.* (24) and were separated from free ^{125}I and degraded hormone by chromatography on a Sephadex G-100 fine columns (Pharmacia) with 0.01 M sodium phosphate buffer, pH 7.5; 0,1% (w/v) bovine serum albumin. The specific activities of ^{125}I-prolactin and ^{125}I-growth hormone were 49 μCi/μg and 76 μCi/μg, respectivetly.

Cell membrane preparations

Mammary glands derived from female rabbits at 15 or 25 day of pregnancy or 5 days of lactation were used for membrane preparation according to Silverstein and Richards (25). One

gram of the frozen tissues was homogenized in 25 mM Tris buffer, pH 7.4 with 150 mM NaCl in an Ultraturrax J25 homogenizer (Janke & Kunkel Labor Technik). Protein content was assayed according to the method Lowry *et al.* (26). The membranes were diluted with the assay buffer (25 mM Tris-HCl, pH7,5; 0,1% BSA, 150 mM sodium chloride) and frozen at −80°C until use for binding assays.

Radioligand assay

Binding and competition assays were performed according to Silverstein and Richards (25). ^{125}I-labelled prolactin or growth hormone (2.5×10^4 to 2×10^5 cpm) was incubated with a sample of the membrane preparation (300 μg of protein) in a final volume 0.5 ml buffer (25 mM Tris-HCl buffer, pH 7.5; 150 mM NaCl and 0,1% BSA) in the presence (or absence) of excess unlabelled hormone (5 μg). After 16-h incubation at room temperature, the reaction mixture was collected on GF/A (Whatman) filters. Radioactivity on the filters was measured in a Beckman gamma 5500 counter with 49% efficiency. Specific binding was obtained by subtracting nonspecific binding (measured in the presence of excess unlabelled hormone) from total binding, and was expressed as a precentage of the total radioactivity added to the incubation.

Specificity of binding of ^{125}I-labelled prolactin to the cell membranes was measured by incubating membrane preparations of the lactating rabbit mammary gland (5 th day of lactation) with ^{125}I-prolactin in the presence of increasing concentrations (0—200 ng/ml) of unlabelled bovine prolactin, bovine or recombinant bovine growth hormone.

Purity of hormones

Preparations of growth hormone and prolactin were analysed by polyacrylamide-SDS electrophoresis. Twenty μg of each hormone were electrophoresed on 15% PAGE-SDS together with a low MW protein marker (Pharmacia). Additionally, the ability of growth hormone to stimulate growth of Nb2 rat lymphoma cells, which have lactogenic receptors, was tested.

Statistical analysis

Statistical calculations were carried out by variance analysis using the SAS General Linear Model.

RESULTS

Effects of prolactin and growth hormone on DNA synthesis

In vivo treatment with prolactin significantly ($p \leq 0.01$) increased (2-fold) the incorporation of the labelled precursor into DNA in mammary gland explants from pregnant rabbits (*Fig. 1*). Injections of recombinant bovine GH also significantly increased DNA synthesis (about 75% in comparison with control animals). The effect of natural GH on DNA synthesis was lower then that of recombinant GH (only 38% above control, nonsignificant).

Effects of prolactin and growth hormone on casein synthesis

The rates of casein synthesis measured by incorporation of [^{3}H] leucine in mammary gland explants from pregnant rabbits in *vivo* treated with hormones are shown in *Fig. 2*. Injections of prolactin strongly enhanced the synthesis of

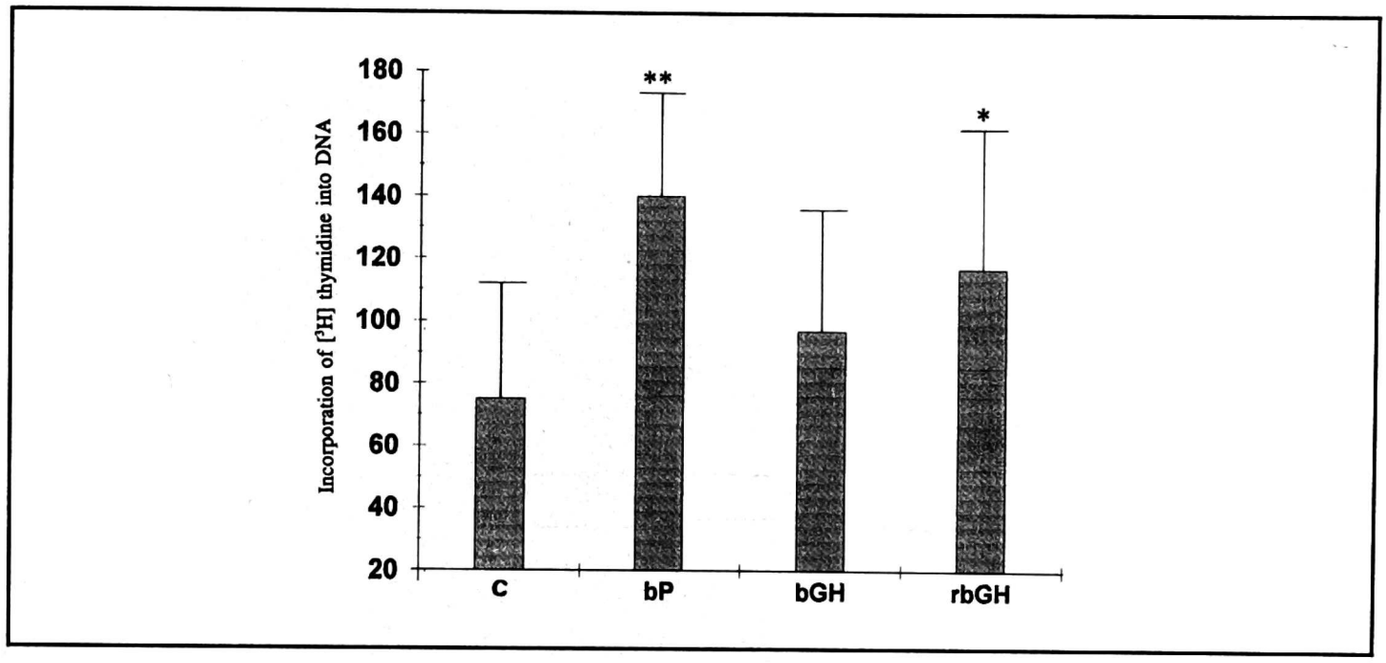

Fig. 1. Effect of bovine prolactin (bP), bovine (bGH) and recombinant bovine (rbGH) growth hormone on DNA synthesis in the rabbit mammary gland.
Mid-pregnant rabbits were injected with hormones. Explants of the mammary gland were prepared and incubated for 4 h at 37°C in Medium 199 containing 1 μCi/ml [^{3}H] thymidine. Each point is the mean ± SEM dpm of triplicate determinations in three separate experiments.
** — $p \leq 0.01$ compared with the corresponding values for control rabbits
C — control (mammary glands of rabbits injected with PBS).

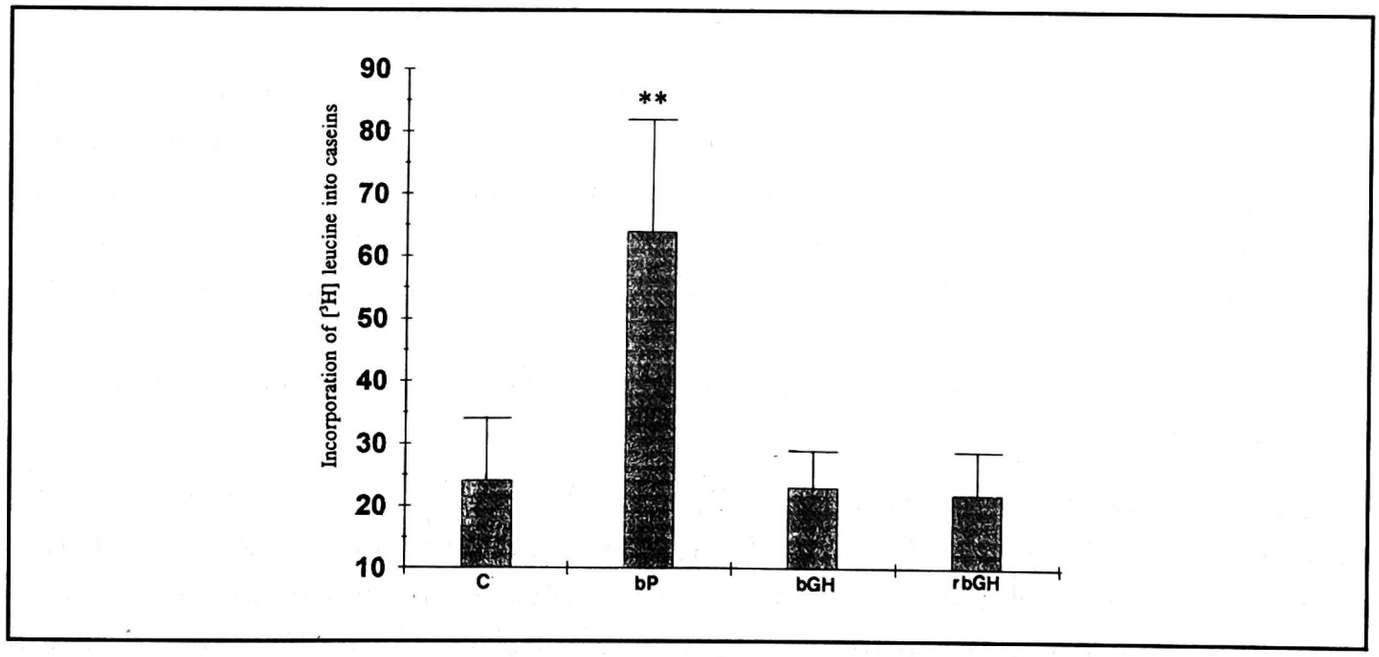

Fig. 2. Effect of bovine prolactin (bP), bovine (bGH) and recombinant bovine)rbGH) hormone on casein synthesis in the rabbit mammary gland.
Mid-pregnant rabbits were injected with hormones. Explants of the mammary gland were prepared and incubated for 4 h at 37°C in Medium 199 containing 1 μCi/ml [^{3}H] leucine. Caseins were precipitated with rennin and calcium. Each point is the mean ± SEM dpm of triplicate determinations in three separate experiments.
** — $p \leq 0.01$ compared with the corresponding values for control rabbits
C — control (mammary glands of rabbits injected with PBS).

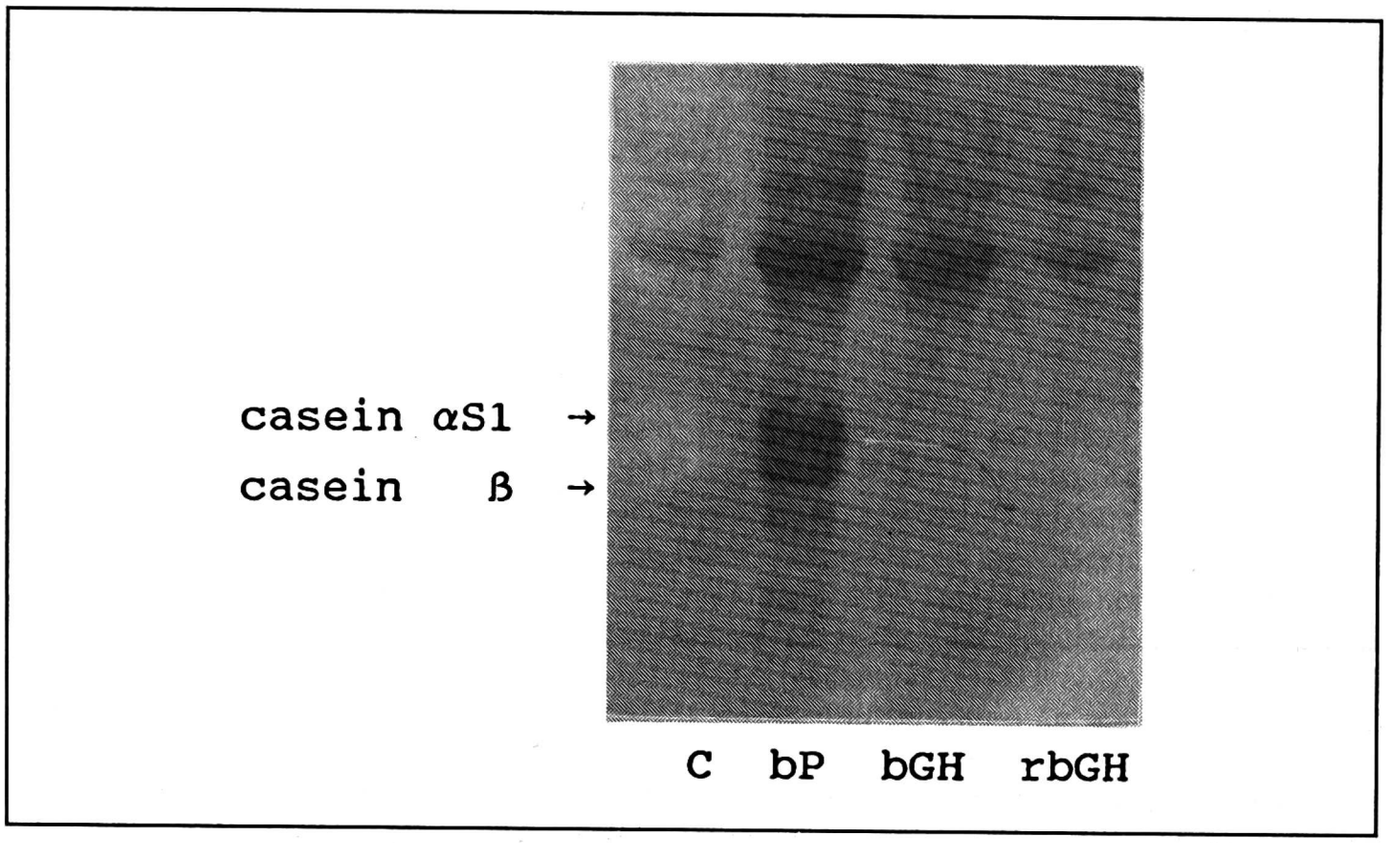

Fig. 3. Effect of bovine prolactin (bP), bovine (bGH) and recombinant bovine (rbGH) growth hormone on the synthesis of αS1 and β caseins in the rabbit mammary gland.
Mid-pregnant rabbits were injected with hormones. Explants of mammary gland were prepared and incubated for 4 h at 37°C in Medium 199 containing 20 μCl/ml [^{35}S] methionine. Caseins precipitated by calcium and rennin were separated on 15% polyacrylamide-SDS gels, transferred to nitrocellulose filters, and autoradiographed.

casein (about 4-fold). Treatments with both natural and recombinant growth hormones had no effect on casein synthesis. Similar results were obtained using [^{35}S] methionine as a precursor of casein synthesis. Only injections of prolactin stimulated synthesis of αS1- and β-casein fractions as shown on the autoradiogram obtained after SDS-polyacrylamide electrophoresis (*Fig. 3*).

Effects of prolactin and growth hormone on the accumulation of αS1- and β-casein and whey acidic protein (WAP) mRNAs.

Steady state accumulation of milk protein mRNAs was measured in the mammary glands of rabbits injected with lactogenic hormones. Representative results are shown in *Fig. 4, 5* and *6*. Injections of prolactin and natural or recombinant growth hormone increased accumulation of αS1- and β-casein and whey acidic protein mRNAs. Prolactin injections were the most effective. Prolactin stimulated accumulation of β- and αS1-casein or WAP mRNAs 13-, 8- and 45-fold, respectively. Recombinant GH was less efficient than natural GH in the stimulation of the expression of milk protein genes. Accumulation of milk protein mRNAs after treatment with pituitary bovine GH was increased 3—6 fold, but with injections of recombinant bovine GH — only 1.5—2 fold above controls.

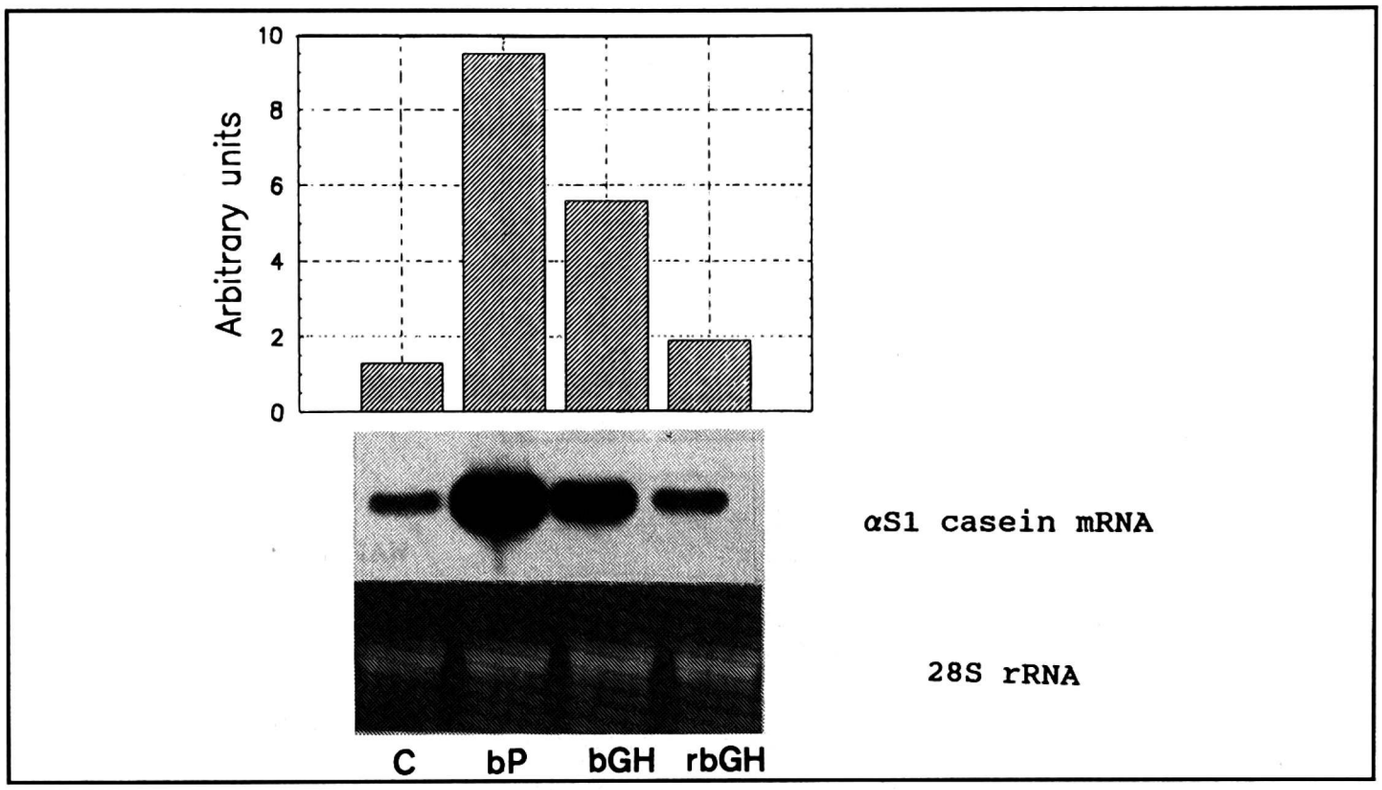

Fig. 4. Effects of bovine prolactin (bP) and bovine (bGH) or recombinant (rbGH) growth hormone on the accumulation of αs1-casein mRNA in mammary glands of pregnant rabbits.
Mid-pregnant rabbits were injected with hormones. The accumulation of αs1-casein mRNA was measured in mammary glands by northern-blot techniques.
C — control (mammary glands of rabbits injected with PBS). Ethidium bromide staining of the gels was used as loading controls.

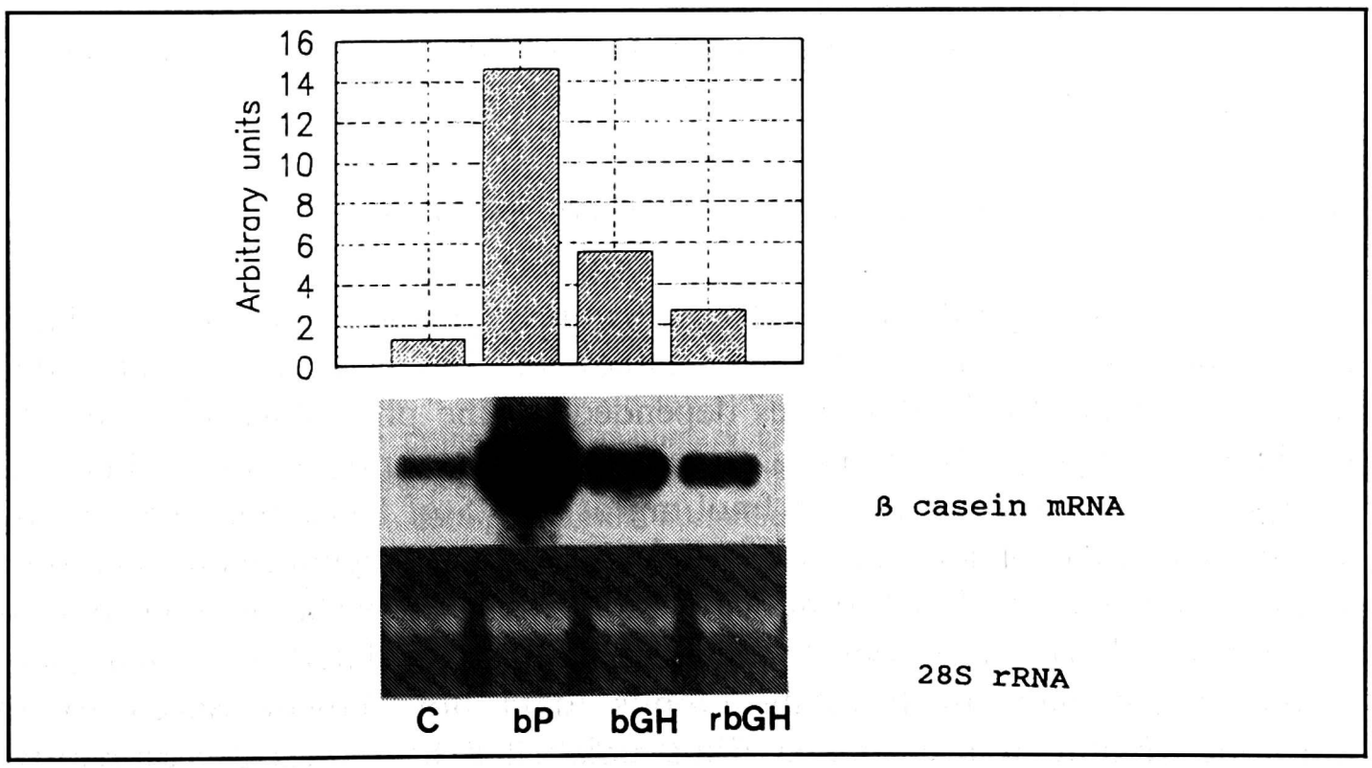

Fig. 5. Effects of bovine prolactin (bP) and bovine (bGH) or recombinant (rbGH) growth hormone on the accumulation of β-casein mRNA in the mammary glands of pregnant rabbits.
Mid-pregnant rabbits were injected with hormones. The accumulation of β-casein mRNA was measured in mammary glands by northern-blot techniques.
C — control (mammary glands of rabbits injected with PBS). Ethidium bromide staining of the gels were used as loading controls.

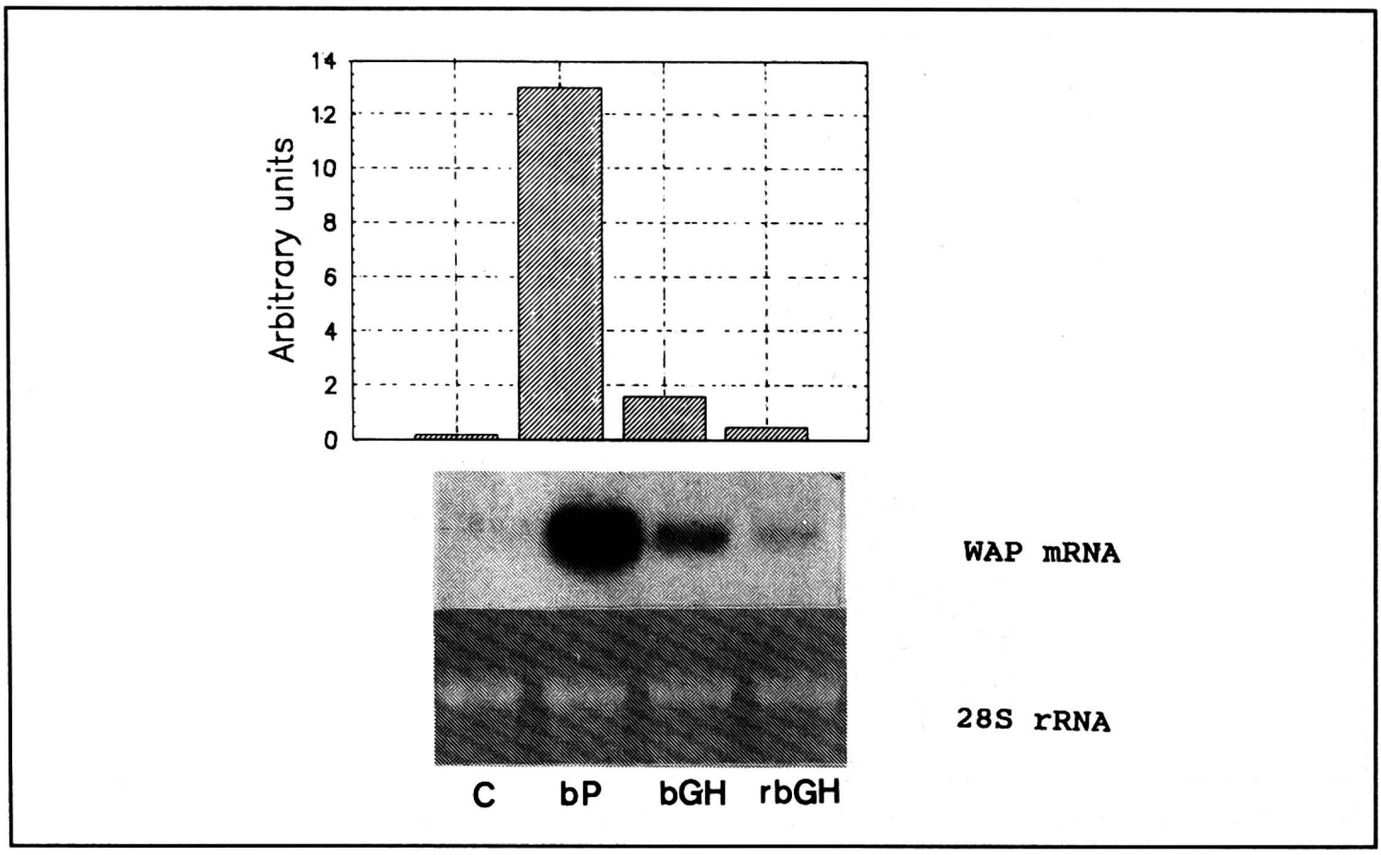

Fig. 6. Effects of bovine prolactin (bP) and bovine (bGH) or recombinant (rbGH) growth hormone on the accumulation of whey acidic protein (WAP) mRNA in the mammary glands of pregnant rabbits.

Mid-pregnant rabbits were injected with hormones. The accumulation of WAP mRNA was measured in mammary glands by northern-blot techniques.

C — control (mammary glands of rabbits injected with PBS). Ethidium bromide staining of the gels were used as loading controls.

Binding of ^{125}I-labelled prolactin and growth hormone

^{125}I-prolactin specifically binds to mammary gland membranes isolated from rabbits at various stages of pregnancy and in lactation. The calculated number of prolactin binding sites depended on the physiological stage. The binding capacity was 18 fmol/mg protein at day 15 of pregnancy, 28 fmol/mg at day 28 of pregnancy and 33 fmol/mg at day 5 of lactation. The binding affinity remaind constant ($K_a - 10^{10}\,M^{-1}$) throughout pregnancy and lactation. These data were calculated from Scatchard plots. No specific binding sites for ^{125}I-labelled bGH have been found in membranes isolated from mammary glands of pregnant or lactating rabbits (data not shown). Specificity of hormone binding was examined using unlabelled hormones as competitive inhibitors of ^{125}I-prolactin (*Fig. 7*). The binding of ^{125}I-labelled prolactin to membrane preparations of the lactating mammary gland was inhibited by increasing amounts of unlabelled bovine prolactin. Natural or recombinant bovine growth hormone did not displace ^{125}I-labelled prolactin from its receptors.

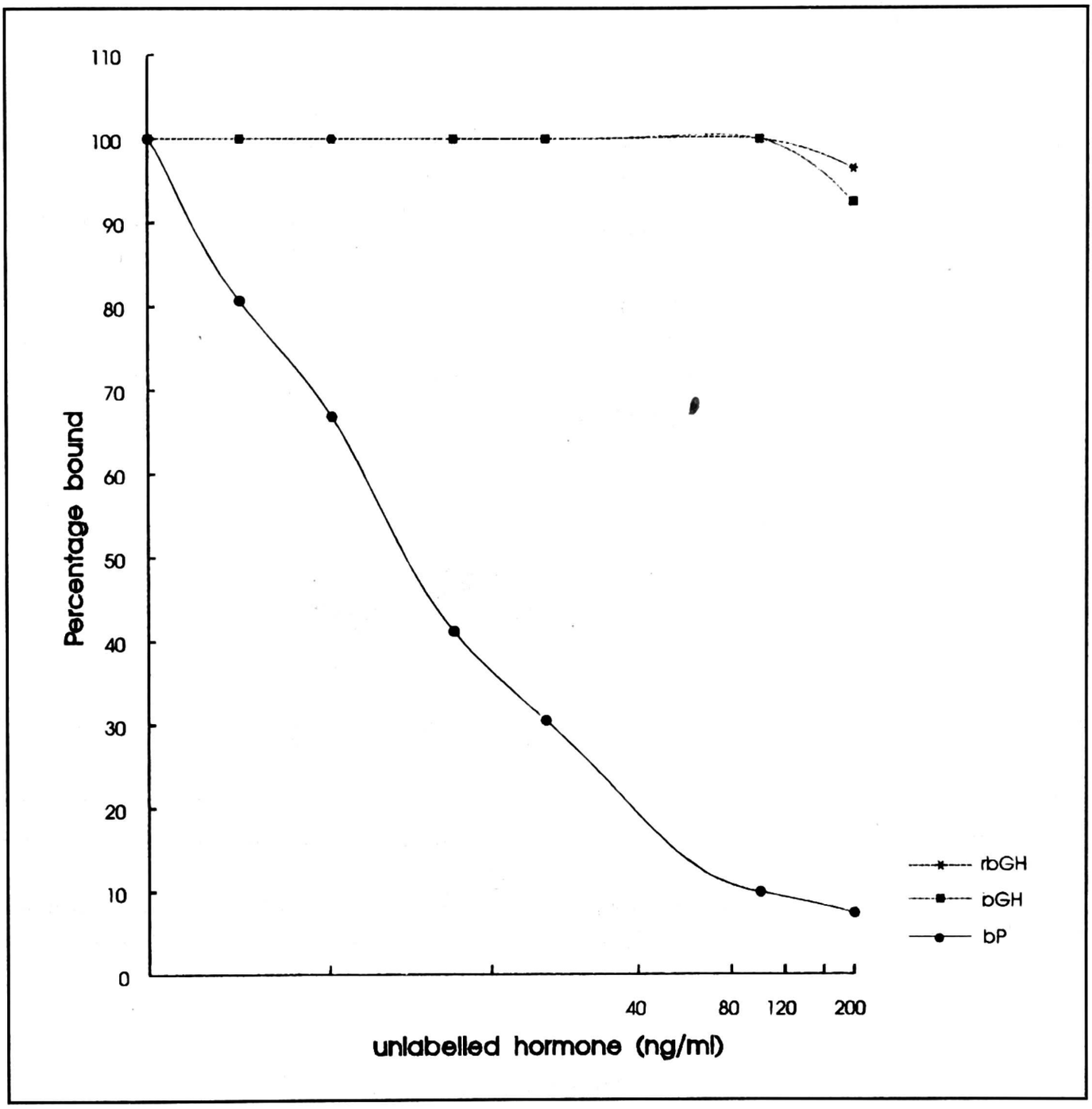

Fig. 7. Competition of prolactin and growth hormone for binding of ^{125}I-labelled prolactin to lactating rabbit mammary gland membrans.
Radioactive prolactin (100 000 cpm) was incubated with samples of membrane preparations and with increasing amounts (0—200 ng/ml) of the hormones indicated: bP — bovine prolactin; bGH — bovine, rbGH — recombinant bovine growth hormones.

Purity of hormone preparations

The purity of hormone preparations used in these experiments was analysed by polyacrylamide-SDS electrophoresis (*Fig. 8*). All preparations appear electrophoreticaly homogenous. In particular GH preparations were shown to be free of prolactin contamination. They also failed to stimulate proliferation of Nb2 lymphoma cells, which is a very sensitive test for prolactin (not shown).

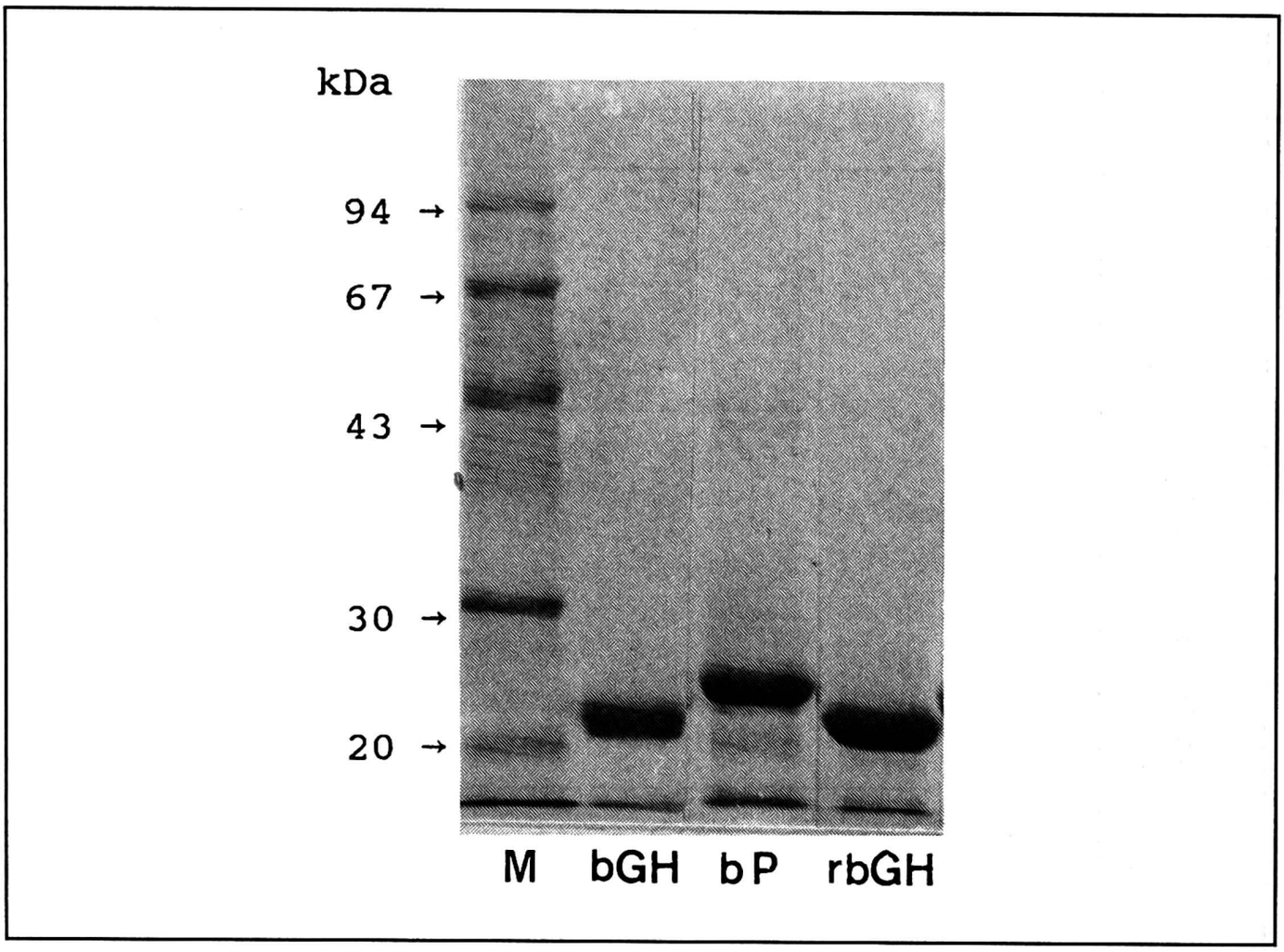

Fig. 8. Analysis of purity of growth hormone and prolactin preparations by polyacrylamide-SDS electrophoresis.
20 μg of bovine prolactin (bP), bovine (bGH) and recombinant bovine (rbGH) growth hormones were electrophoresed on 15% polyacrylamide-SDS gel together with low molecular weight protein marker (M).

DISCUSSION

Prolactin injections into mid-pregnant rabbits induced DNA synthesis in the mammary glands. Our results confirm earlier findings by Houdebine *et al.* (27) showing that prolactin injected into pseudopregnant rabbits stimulated DNA synthsis and those of Zwierzchowski *et al.* (19) showing that prolactin injections into virgin rabbits increased both mammary gland DNA synthesis and DNA polymerase-α activity. In our experiments recombinant bovine GH (rbGH) also stimulated DNA synthesis. Stimulation of DNA synthesis by natural bovine growth hormone was lower than that by prolactin or rbGH. Our results showed that in the rabbit mammary gland both prolactin and GH are mammogenic; they stimulate mammary gland growth. Different results were obtained from experiments conducted in ruminant mammary glands. In cows and goats injections of bovine GH did not increase DNA synthesis (28, 29).

Of the hormones tested in our experiments only prolactin significantly stimulated casein synthesis as measured by incorporation of [^{3}H] leucine or [^{35}S] methionine into casein fractions. Growth hormone had no effect on casein synthesis. Caron *et al.* (30) studied the capacity of natural or recombinant GH preparations from different species to replace prolactin in the induction of lactogenesis in pregnant rats. They showed that both natural and recombinant human GH are potent inducers of milk protein synthesis. Also rat and ovine GH had a partial activity in inducing casein synthesis. In these experiments porcine and bovine GHs were ineffective. Studies by Feldman *et al.* (31) showed that „nonlactogenic" rat GH was far more potent than prolactin in inducing rat mammary gland development. Recently, Flint and Gardner (32) reported that in lactating rats bovine growth hormone exerted direct *in vivo* effect on the mammary gland growth and milk protein synthesis.

In the present work we have shown that pituitary and recombinant bovine growth hormone could induce the expression of milk protein genes — caseins αS1 and β and whey acidic protein — in mammary glands of pregnant rabbits. Both natural and recombinant GHs were less effective than prolactin and the activity of recombinant GH in inducing expression of milk protein genes was less than that of natural bovine GH. Our results showed that *in vivo* treatment with prolactin increased both transcription of milk protein genes and casein synthesis. Growth hormone seems to act only at the stage of milk protein gene transcription and is not able to induce casein synthesis.

In order to show the specificity of the effect of GH on the expression of milk protein genes we tested electrophoretically the purity of hormone preparations used in these experiments. We showed that GH preparations were free of prolactin contamination. The GH preparations also failed to stimulate proliferation of Nb2 rat lymphoma cells (this test detects prolactin at pg concentrations). Moreover, also recombinant bGH, which obviously cannot be contaminated by prolactin, was shown to be active in the stimulation of both mammary DNA synthesis and milk protein gene expression. These data support the hypothesis that GH could specifically act on the mammary gland cells.

The initial step in the action of peptide hormones is binding to receptors in target tissues. Prolactin receptors are abundant in the rabbit mammary gland but GH receptors have not yet been detected. Our results showed that prolactin receptors in rabbit mammary gland cells increased from pregnancy to lactation and their abundance was comparable to that measured by Djiane *et al.* (10). However, we were unable to detect binding sites for ^{125}I-labelled bGH in membranes isolated from mammary glands of pregnant or lactating rabbits.

Prolactin receptors belong to growth hormone/prolactin/cytokine receptors family (33). Growth hormone receptor of rabbit liver has been seqenced and cDNA encoding growth hormone receptor has been cloned (34). Specific

bindings sites for GH in mammary gland membranes have not yet been characterized by conventional assays. Although mRNA for growth hormone receptor has been detected in several species, including rabbits, it is not known whether receptor protein is expressed. Another possible mechanism of GH actions on mammary gland growth and gene expression may be through its binding to prolactin receptors. However, we have shown that GH was unable to displace prolactin from its mammary receptors. These results suggest that the mechanism of action of GH does not involve a lactogenic receptor.

Our results shown that growth hormone separately from prolactin may directly regulate expression of casein and whey protein genes in the rabbit mammary gland. However, the mechanism of its action is still unknown.

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