Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 5125–5130, May 1997
Developmental Biology

Functional growth hormone (GH) receptors and GH are expressed by preimplantation mouse embryos: A role for GH in early embryogenesis?

(preimplantation physiology/protein synthesis/glucose transport)

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Communicated by Henry G. Friesen, Medical Research Council of Canada, Ottawa, Canada, March 3, 1997 (received for review March 1, 1996)

ABSTRACT **The results of this study challenge the widely held view that growth hormone (GH) acts only during the postnatal period. RNA phenotyping shows transcripts for the GH receptor and GH-binding protein in mouse preimplantation embryos of all stages from fertilized eggs (day 1) to blastocysts (day 4). An antibody specific to the cytoplasmic region of the GH receptor revealed receptor protein expression, first in two-cell embryos, the stage of activation of the embryonic genome (day 2), and in all subsequent stages. In cleavage-stage embryos this immunoreactivity was localized mainly to the nucleus, but clear evidence of membrane labeling was apparent in blastocysts. GH receptor immunoreactivity was also observed in cumulus cells associated with unfertilized oocytes but not in the unfertilized oocytes. The blastocyst receptor was demonstrated to be functional, exhibiting the classic bell-shaped dose–response curves for GH stimulation of both 3-*O*-methyl glucose transport and protein synthesis. Maximal stimulation of 40–50% was seen for both responses at less than 1 ng/ml recombinant GH, suggesting a role for maternal GH. However mRNA transcripts for GH were also detected from the morula stage (day 3) by using reverse transcription–PCR, and GH immunoreactivity was seen in blastocysts. These observations raise the possibility of a paracrine/autocrine GH loop regulating embryonic development in its earliest stages.**

Embryonic and fetal growth have long been considered to be independent of pituitary growth hormone (GH). However, this view is challenged by a growing body of evidence which demonstrates a role for GH in the development of the fetus. Newborn Laron dwarfs, lacking a functional GH receptor, are more than 2 SD shorter than normal (1). Exogenous GH has been shown to restore embryonic growth in rats after transplantation of parts of embryos into hypophysectomized hosts (2). A number of fetal tissues has been shown to respond to GH *in vitro* (3–6). GH receptor transcripts have been demonstrated in day 12 rat embryos and placentae (7), day 51 sheep embryos (8), and mouse placenta (9). Immunoreactive GH receptor was observed in the human fetus from the second trimester (10, 11). Recently GH receptor transcripts and immunoreactivity have been demonstrated in germ-line competent mouse embryonic stem cells, and GH receptor transcripts were also demonstrated in mouse blastocysts (12).

Preimplantation stages earlier than the blastocyst, however, were not examined by Ohlsson *et al.* (12). Furthermore, there was no evidence that these very early embryos were capable of receptor synthesis or of signal transduction after ligand binding to expressed receptor. In this study we demonstrate the presence of GH receptors in the early embryo from fertilization to the blastocyst stage and the ability of GH to influence the metabolism of blastocyst. Moreover, we report the expression of GH by preimplantation blastocysts, raising the possibility of paracrine/autocrine regulation of embryonic growth by GH.

MATERIALS AND METHODS

Gene Expression. Fertilized eggs, two-cell embryos, morulae, and blastocysts were collected [24, 48, 72, and 96 hr after administration of 10 international units of human chorionic gonadotropin (hCG)] from mated, superovulated Quackenbush mice. RNA obtained by using extraction with phenol/chloroform and precipitation with ethanol (13) was reverse transcribed by oligo(dT) priming and avian myeloblastosis virus reverse transcriptase (GIBCO/BRL). The cDNA derived from the equivalent of total RNA from at least 10 embryos was used in PCRs to specifically amplify cDNAs of interest (13). The PCR products were resolved on 2% agarose gels containing 0.5 μg/ml ethidium bromide. cDNA samples were first tested, and discarded if found to be contaminated with genomic DNA. This was determined by PCR with a primer pair for mouse β-actin which gives a predicted 243-bp fragment for the cDNA and a 330-bp fragment (due to presence of an intron) if contaminating genomic DNA is present (14). Primer pairs used in the PCR reaction were derived from published mouse sequences. These and the sizes of the expected PCR fragments are shown in Table 1. To confirm identity, PCR products were sequenced on an Applied Biosystems 373A DNA sequencer.

Immunolocalization. Embryos were fixed in 2% paraformaldehyde in phosphate-buffered saline, pH 7.4 (PBS), for 30 min at 25°C, and then washed four times in PBS before being placed on Cell-Tak (Collaborative Research) -coated chambers for further processing. Embryos were permeabilized with 0.25% Triton X-100 (Sigma) in PBS for 15 min at 25°C, washed with PBS, neutralized with 0.05 M NH_4Cl/PBS for 10 min, washed, and then incubated in a blocking solution containing 10% normal goat serum (NGS), 5 g/liter BSA, and 0.01% Tween-20 in PBS (NGS/BSA/Tween/PBS) for 45–60 min. Blocked embryos were then incubated with primary antibody diluted in 5% NGS/BSA/Tween/PBS for 2 hr at 25°C. After

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0027-8424/97/945125-6$2.00/0
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Abbreviations: GH, growth hormone; bGH, bovine GH; hCG, human chorionic gonadotropin; hGH, human GH; GST, glutathione *S*-transferase; 3-OMG, 3-*O*-methyl-D-glucose; IGF-1, insulin-like growth factor 1.

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Table 1. GH, GH receptor, and GH-binding protein PCR primer sequences

Gene	Primer	Sequence	Nucleotides in cDNA	Fragment size, bp
GH	5′	5′-CAGCCTGATGTTTGGTACCTCGGA-3′	433–456	253
	3′	5′-GCGGCGACACTTCATGACCCGCA-3′	663–685	
GH receptor	5′	5′-AGTTGGAGGAGGTGAACACCAT-3′	980–1001	330
	3′	5′-GGCACAAGAGATCAGCTTCCAT-3′	1288–1309	
GH binding-protein	5′	5′-AAGTACAGCGAGTTCAGCGAAGTC-3′	739–762	146
	3′	5′-CGGATCCTCTGAAGCTGGTGATA-3′	862–884	

PCR primer sequences were designed from published mouse sequences: GH (15) and GH receptor and GH-binding protein (16).

primary antibody incubation, the embryos were washed in PBS and then exposed to Texas red-conjugated secondary antibody diluted 1:100 in PBS for 1 hr at 25°C. Coverslips were mounted on cavity slides in PBS-buffered glycerol and examined using a Bio-Rad MRC-600 confocal laser scanning microscope mounted on a Zeiss Axioskop equipped with a Zeiss Plan-Apochromat ×63 oil-immersion objective.

Antibodies. The GH receptor antibody was a polyclonal antiserum raised against a pGEX fusion protein of the entire rabbit GH receptor cytoplasmic region from phenylalanine-258 fused to glutathione *S*-transferase (GST) and characterized in the rat as previously described (17). The monkey anti-rat GH antiserum was obtained from A. F. Parlow (Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA) and was developed using highly purified rat GH as the immunogen. Secondary antibodies used in immunofluorescence were Texas red-conjugated goat anti-rabbit and goat anti-human IgG for the GH receptor and GH studies, respectively (Calbiochem–Novabiochem).

Effect of GH on 3-*O*-Methyl-D-glucose (3-OMG) Uptake. Glucose transport was measured directly using 3-*O*-methyl-D-[1-^{3}H]glucose (^{3}H-3-OMG; Amersham). Blastocysts collected 96 hr after administration of hCG were incubated in M2 medium (18) modified as previously described (19), containing 0–30 ng/ml recombinant bovine GH (bGH; gift of Monsanto Chemical Co. St Louis) at 37°C in a humidified atmosphere of 5% CO_2/5% O_2/90% N_2. After 60 min of culture, they were transferred to 10-μl droplets of glucose-free M2 containing 0.3 mM (37 GBq/liter) ^{3}H-3-OMG and 25 mM 3-OMG (Sigma) and incubated at 37°C for 3 min. Uptake of ^{3}H-3-OMG was stopped by transferring the embryos in a minimal volume of medium through a series of four washes each of 2 ml of ice-cold glucose-free M2. Embryos were counted individually in a Packard 2500TL liquid scintillation counter at 40% efficiency with a background of 4–5 cpm. The experimental background was not different from this absolute background.

Effect of GH on Protein Synthesis. Blastocysts were cultured in BMOC2 (ref. 20; modified as previously described in ref. 21) at 37°C in a humidified atmosphere of 5% CO_2/5% O_2/90% N_2 for 4 hr with 0–100 ng/ml human GH (hGH; gift of Genentech Inc., San Francisco) or recombinant bGH before transfer to 20-μl droplets of the same medium containing 6 μM (37 GBq/liter) [4,5-^{3}H]leucine (Amersham). After 2 hr at 37°C, blastocysts were transferred through four washes of ice-cold BMOC, placed individually into scintillation vials containing 100 μl of BMOC, and frozen. After rapid thawing, 100 μl of 20% trichloroacetic acid (TCA) was added and protein which precipitated at 4°C over 2 hr was collected on glass fiber discs (Whatman GFC, 24 mm) and washed with 10% TCA to remove acid-soluble material (22). This incorporation of [^{3}H]leucine into acid-precipitable protein, assayed as above, is referred to as measuring protein synthesis.

Statistical Analysis. STATGRAPHICS (Version 3.0, Manugistics, Rockville, MD) was used for ANOVA and multiple means range tests using Fisher's protected procedure.

RESULTS

Gene Expression. To develop an mRNA phenotypic map for the expression of the GH gene family in early mouse embryos, reverse transcription (RT)-PCR studies were carried out with primer pairs specific for GH, GH receptor, and GH-binding protein. GH receptor and GH-binding protein transcripts were detectable in all stages of preimplantation embryo development examined, from the one-cell to blastocyst stages (Fig. 1). They showed an expression pattern typical of many genes that are constitutively expressed during early development: strong expression in the fertilized egg, decreased abundance at the two-cell and morulae stages due to degradation of maternal mRNA, and re-accumulation in blastocysts due to new transcription from the embryonic genome (23). Under the conditions utilized in these studies, ethidium bromide stained bands of RT-PCR products for GH mRNA were not detectable until the morula stage of development (day 3) (Fig. 1). A mouse pituitary cDNA sample derived from the equivalent of 20 ng of total mRNA was included as a positive control for GH and GH receptor, 3T3-F442A pre-adipocytes were used as a positive control for GH-binding protein, while cDNA from non-expressing Chinese hamster ovary (CHO) cells was used as a negative control for all three mRNAs (Fig. 1 *Bottom*).

Immunolocalization. Using an antiserum that recognizes the carboxyl terminus of GH receptor (17) and hence the full-length receptor, immunoreactivity was observed from the two-cell stage of development through to the blastocyst (Fig. 2). There was no apparent staining in either fertilized or unfertilized eggs, although positive staining was observed on cumulus cells surrounding the unfertilized oocytes (Fig. 2*B*). The specificity of this immunoreactivity was demonstrated by the absence of staining in embryos incubated with preimmune serum (Fig. 2*G*), and with antibody preabsorbed with 20 μg/ml rabbit GH receptor [purified according to Spencer *et al.* (24)] (Fig. 2 *I* and *J*)]. This contrasted with the retention of immunoreactivity when the antiserum was preincubated with 20 μg/ml purified recombinant GST for the same time (24 hr, 4°C; Fig. 2*H*). Positive staining in these early embryos appeared localized to the nucleus but not in the nucleoli, with some staining apparent on the plasma membrane in two-cell embryos.

GH immunoreactivity was observed in both freshly collected and *in vitro* cultured mouse blastocysts when an antiserum directed against the rat hormone was used (Fig. 3). This immunoreactivity, which was attenuated by preabsorption with rat GH, was predominantly localized to outer membranes of trophectoderm cells, with some immunoreactivity in nuclei of freshly collected blastocysts. In culture-derived blastocysts GH immunoreactivity was apparent in cytoplasmic vesicles proximal to nuclei (possibly trans-Golgi).

Glucose Transport. Blastocysts responded to a 60-min exposure to bGH with increased glucose transport (Fig. 4). bGH stimulated glucose transport, with maximal stimulation of 44% ($P < 0.01$) occurring at 0.1 ng/ml (4.5 pM) bGH. Higher concentrations of GH resulted in a gradual decline in transport activity to basal levels at 100 μg/ml.

Protein Synthesis. The incorporation of [^{3}H]leucine into acid-insoluble material by mouse blastocysts was increased by

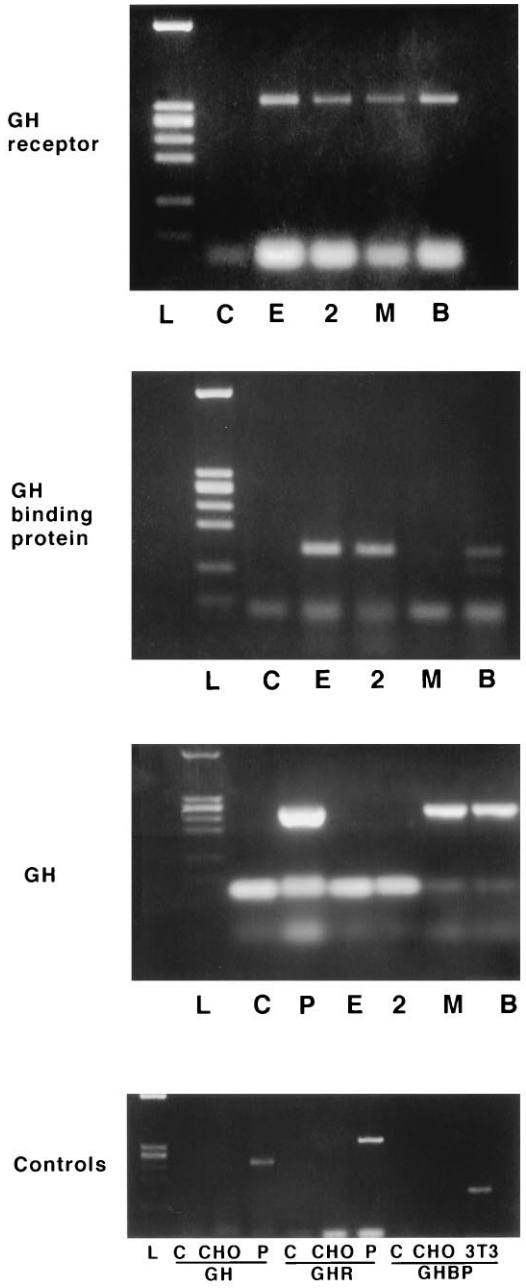

FIG. 1. Expression of GH, GH receptor, and GH-binding protein transcripts in early mouse embryos. The sample in each lane was produced by using a cDNA aliquot derived from RNA from the equivalent of 10 embryos. The RNA preparations were reverse transcribed and amplified by 40 cycles of PCR using gene-specific oligonucleotides described in Table 1. Lanes are L, DNA ladder (bands from top to bottom: 603 bp, 310 bp, 294/281 bp, 234 bp, 194 bp, 118 bp, and 72 bp); C, negative control (no cDNA); CHO, Chinese hamster ovary cells (negative control); E, fertilized eggs; 2, two-cell embryos; M, morulae; B, blastocysts; P, pituitary [GH- and GH-receptor (GHR)-positive control]; 3T3, 3T3-F442-A fibroblasts [GH-binding protein (GHBP)-positive control].

a maximum of approximately 50% ($P < 0.701$) in response to hGH (Fig. 5) and 40% ($P < 0.01$) in response to bGH. The lowest statistically significant stimulation was at 0.1 ng/ml (4.5 pM) hGH and bGH. The response to hGH showed a marked decrease at 100 ng/ml which was not apparent with bGH.

DISCUSSION

This study investigated the expression and function of GH, GH receptor, and GH-binding protein during mouse preimplantation development. Our results show that GH receptor and GH-binding protein transcripts are present in embryos at all stages of preimplantation development. Furthermore, in blastocysts these receptors are functional, transducing the ligand-binding event to stimulate glucose transport and protein synthesis. The presence of functional GH receptors at this early stage raises the possibility that GH may be involved in early embryonic growth and development.

While GH receptor transcripts were present from the fertilized oocyte and throughout all preimplantation stages, GH receptor immunoreactivity was not apparent until the two-cell stage. This difference in ontogeny may be a function of the acute sensitivity of reverse transcription–PCR as opposed to immunohistochemistry or, alternatively, a result of GH receptor translation not occurring until the two-cell stage. Histo-

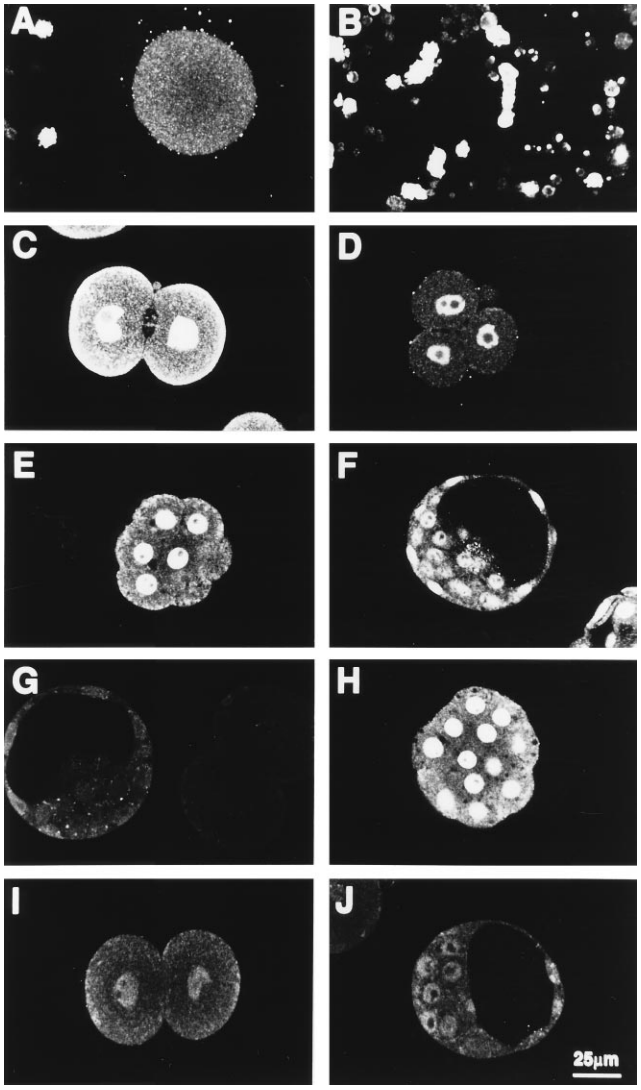

FIG. 2. Ontogeny of GH receptor immunoreactivity in preimplantation mouse embryos. Shown are confocal images of optical sections of the following. (*A–F*) Fertilized oocyte (*A*), cumulus cells (*B*), two-cell embryo (*C*), four-cell embryo (*D*), morula (*E*), and blastocyst (*F*) incubated with GH receptor antiserum. (*G*) Two-cell embryo and blastocyst incubated with preimmune serum. (*H*) Morula incubated with GH receptor antiserum preabsorbed with 20 μg/ml GST. (*I* and *J*) Two-cell embryo and blastocyst incubated with GH receptor antiserum preabsorbed with 20 μg/ml rabbit GH receptor. Note positive immunoreactivity appears on cumulus cells surrounding ovulated oocytes and is localized to the nuclei of cleavage-stage embryos and blastocysts. Consistent staining was observed in at least three experiments in which a total of 150 embryos were surveyed.

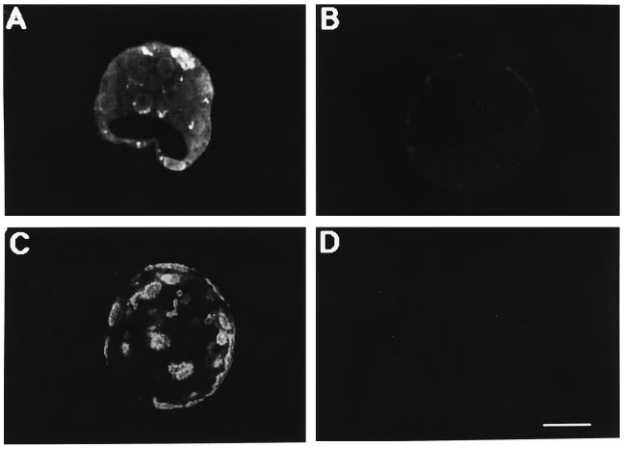

FIG. 3. Confocal images of mouse blastocysts showing positive immunoreactivity for GH. Blastocysts were incubated with monkey anti-rat GH antiserum (*A* and *C*), nonimmune monkey serum (*D*), or GH antiserum preabsorbed with 20 μg/ml rat GH (*B*). In a reconstructed three-dimensional image of a blastocyst (*C*) positive immunoreactivity appears on the outer membranes of the trophectoderm, with some immunoreactivity apparent in nuclei. In optical sections (*A*) the immunoreactivity is also apparent in cytoplasmic perinuclear vesicles. Consistent staining was observed in three experiments in which a total of 60 blastocysts were surveyed. (Bar = 25 μm.)

chemical preparation of the oocyte material was effective, since GH receptor immunoreactivity was observed in cumulus cells associated with the ovulated oocytes. This finding is consistent with an indirect role for GH in oocyte maturation, as suggested by Apa *et al.* (25).

The nuclear staining observed in these early embryos is compatible with previous work demonstrating GH receptor association with the nucleus in rat and rabbit tissues (17, 26). The functional significance of a nuclear GH receptor, while currently unclear, is not without precedent. Other polypeptide

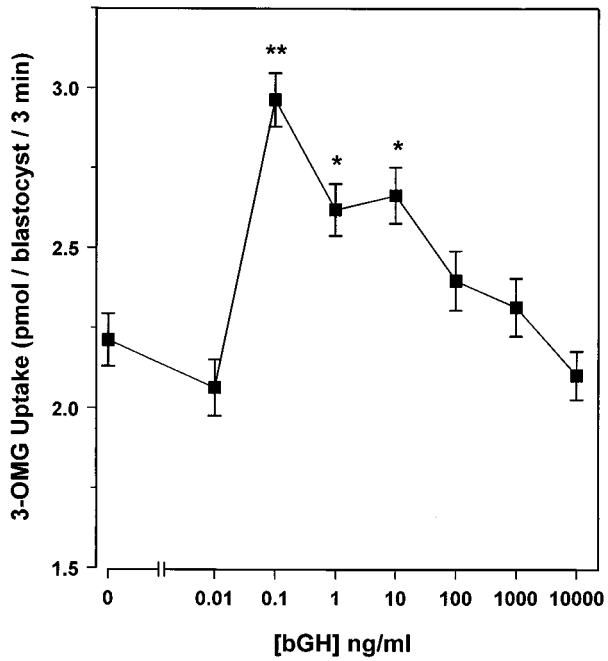

FIG. 4. Effect of bGH on blastocyst glucose transport. Blastocysts were incubated with 0–10,000 ng/ml (0–410 nM) bGH for 60 min, and then the uptake of 25 mM ^{3}H-3-OMG was measured over 3 min at 37°C. Values represent the mean ± SEM of three experiments, each including 4–10 blastocysts per point. **, $P < 0.01$; *, $P < 0.05$ in comparison with 0 ng/ml bGH by ANOVA.

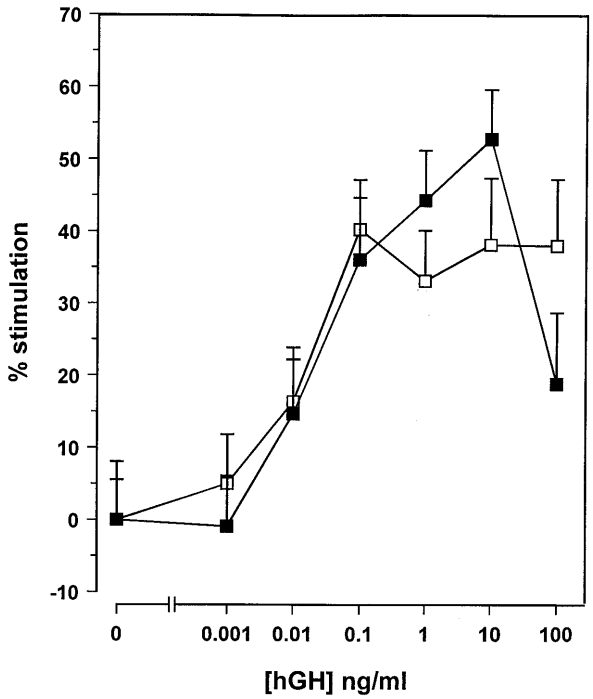

FIG. 5. Effect of hGH (■) and bGH (□) on blastocyst protein synthesis. Blastocysts were cultured for 4 hr with 0–100 ng/ml (0–4.1 nM) hGH or bGH before the incorporation of [^{3}H]leucine into acid-precipitable protein over 2 hr was assayed as described in the text. Each point represents the mean ± SEM of three experiments, of the percentage increase from control, with each experiment including 3–9 blastocysts per treatment group (mean control values for hGH and bGH experiments are 25,314 ± 2,036 and 18,477 ± 1,020 cpm per blastocyst per 2 hr, respectively). For GH concentrations >0.01 ng/ml; $P < 0.01$ in comparison with 0 ng/ml GH for each set of experiments, with the exception of 100 ng/ml hGH treatment, by ANOVA.

hormone receptors have been found to associate with the nucleus, including insulin (27), prolactin (28), epidermal growth factor, nerve growth factor, platelet-derived growth factor (29, 30), and fibroblast growth factor (31).

That the blastocyst receptor is functional is clearly demonstrated by the actions of GH on cellular functions involving classic early insulin-like effects of GH. GH significantly stimulated glucose uptake and protein synthesis by blastocysts at the very low concentrations likely to be present *in vivo*. The potencies of hGH and bGH are similar in that maximal stimulation is first apparent at 4.5 pM for both hormones. This agrees with the relative potencies of these ligands for the nonprimate GH receptor (32). Furthermore the dose response for glucose uptake clearly shows down-regulation of transport activity with increasing GH concentrations. This is characteristic of many GH dose–response curves and is thought to be due to the blocking of GH receptor dimerization by high GH concentrations, which sequester all receptor molecules in site 1 interactions (33, 34).

The appearance of GH transcripts just 72 hr after fertilization, and GH immunoreactivity in the blastocyst (day 4) raises intriguing questions about the mode of possible GH action at this stage of development. While some of the GH immunoreactivity observed in blastocysts may be of maternal origin, the presence of positive GH immunoreactivity in perinuclear cytoplasmic vesicles suggests that this is of embryonic origin and may be involved in development. Prior to this discovery, we presumed that maternally derived GH or an autocrine/paracrine GH-like peptide from the embryo would be the ligand for the embryonic receptors. While the placenta does produce placental lactogens (35, 36) and even GH variants in some species (37), the presence of these peptides prior to

placentation, in particular in preimplantation embryos, has not been established. Placental GH variants might be of greater importance following implantation, since placental lactogen, at least, is not synthesized at high concentrations by the placenta until about day 7 (38), well before pituitary GH can be detected [around day 17 (7, 38)]. Nonetheless, our results show that a purely embryonic autocrine/paracrine mode for GH action must now be considered. An endocrine role for *pituitary* GH in prenatal growth has been rejected in the past. Possibly embryonic production of GH is more important to the early embryo, since expression of GH and functional GH receptor provides all the components of a potential autocrine/paracrine regulatory pathway in the very early embryo, and the hormone concentrations required to produce a maximal response are suitably low. In view of the presence of functional GH receptor so early in development, it is difficult to accept the dogma that GH has no role in prenatal growth.

Embryonic GH could influence the proliferation and/or differentiation of embryonic cells as well as modulating embryo metabolism. It is well established that the preimplantation embryo expresses receptors for and responds to a number of growth factors *in vitro* (39).

It is not clear if the actions of GH on the blastocyst reported here represent direct effects of GH or are mediated by insulin-like growth factor 1 (IGF-1), which is also expressed by preimplantation embryos and whose receptor first appears in morulae in the mouse (40). Since GH stimulates the synthesis and secretion of IGF-1 in many tissues and autocrine/paracrine actions of IGF-1 are believed to be important in the local effects of GH on these tissues (41), it is tempting to speculate that GH regulates growth and differentiation of the early embryo by means of stimulation of IGF-1. While it is not known if GH can influence the synthesis and secretion of IGF-1 by preimplantation embryos, all the elements of an IGF-1 autocrine/paracrine system have been demonstrated in the blastocyst—i.e., expression of IGF-1 (40, 42), IGF-1 receptor (40), and IGF-binding proteins (43). Furthermore IGF-1 has a number of metabolic and proliferative effects on blastocysts, including increasing the rates of inner cell mass proliferation, blastocyst formation (44), protein synthesis (45), endocytosis (46), and glucose transport (47) under conditions identical to those used in this study. Indeed the degree of maximal stimulation of glucose transport by GH and IGF-1 are very similar and within the same time frame (47).

However, in view of the limited time of exposure to GH in our glucose transport study (1 hr), a direct effect by GH seems more likely and would be congruent with recent evidence that GH addition induces rapid tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) and mitogen-activated protein kinases (48). GH effects on glucose transport were recently shown to be due to direct recruitment of the transporters GLUT1 and GLUT4 to the plasma membrane (49). As GLUT1 is the only recruitable transporter present in blastocysts (50, 51), it follows that the increase in glucose transport observed in our study is due to direct GH stimulation of GLUT1 recruitment/synthesis.

In summary, we have shown that GH receptors are among the earliest receptors expressed on preimplantation mouse embryos, while GH is first expressed at the morulae stage. GH receptor immunoreactivity was associated with the nucleus in cleavage-stage embryos, while in the blastocyst it was also distributed throughout the cytoplasm and plasma membrane. Low concentrations of GH stimulated protein synthesis and glucose transport in blastocysts, with both responses showing the characteristic bell-shaped dose–response curve associated with GH action as seen in proliferation assays (34). These responses are likely to represent direct affects of GH, although we cannot eliminate involvement of autocrine/paracrine IGF-1. We suggest that GH should be considered as an additional element in a complex and dynamic system in which embryo–maternal communications are important for viability of the embryo. The actions of GH on the very early embryo add another dimension both to GH actions and to the control of early development.

We thank Katrina Chambers for excellent technical assistance, Dr. A. F. Parlow of the National Institute of Diabetes and Digestive and Kidney Diseases for the GH antiserum, and Colin MacQueen for his assistance with the confocal laser scanning microscope. The Confocal Microscopy Facility of the University of Queensland was supported by a grant from the Australian Research Council. M.P. was supported by a Postgraduate Re-entry Research Scholarship from The University of Queensland. Part of this work was supported by separate grants from National Health and Medical Research Council to M.J.W. and P.L.K.

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