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Possible roles of insulin and insulin-like growth factors in rat preimplantation development: investigation of gene expression by reverse transcription–polymerase chain reaction

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The sensitive mRNA phenotyping technique of reverse transcription–polymerase chain reaction was used to demonstrate that insulin receptor mRNA is present in rat embryos during the preimplantation period. In addition, mRNA encoding insulin-like growth factor (IGF) type I and type II receptors have also been detected in rat preimplantation embryos. IGF-I mRNA was not detected in preimplantation embryos but was found in oviducts and uteri of prepubertal and early pregnant rats. IGF-II mRNA was present in both embryos and in oviducts and uteri during the preimplantation period. These findings suggest that insulin and IGF-I could influence early embryo development in endocrine or in paracrine fashions, whereas IGF-II may have an additional autocrine mode of action in affecting preimplantation embryos in rats.

Introduction

Early embryonic mortality is a significant contributor to pregnancy failure in many mammalian species (Hafez, 1967; Cooke, 1988). Prevention of early embryonic mortality requires a thorough understanding of the regulation of embryo development. Evidence is accumulating that factors promoting the growth of various types of tissue and cell may also control the growth of early mammalian embryos (Harvey and Kaye, 1988; DeChiara *et al.*, 1990; Paria and Dey, 1990). We previously reported that the addition of insulin to the culture medium improves the development of rat preimplantation embryos *in vitro*, and that this action of insulin requires the presence of amino acids (Zhang and Armstrong, 1990). The presence of insulin receptors has been demonstrated in eight-cell mouse embryos (Heyner *et al.*, 1989). In addition, mRNAs encoding insulin-like growth factor (IGF) type I and type II receptors and IGF-II are also present in preimplantation embryos from mice (Rappolee *et al.*, 1990) and cattle (Watson *et al.*, 1992), suggesting that these receptors may transmit possible embryotrophic actions of insulin and IGFs.

A sensitive procedure of reverse transcription–polymerase chain reaction (RT–PCR) was used to examine the presence or absence of mRNA encoding insulin receptors in rat preimplantation embryos to investigate whether the previous observed beneficial effect of insulin on rat embryos was mediated by

specific receptor binding. The gene transcripts of IGFs I and II and their receptors were also examined for their possible presence in rat embryos and oviducts and uteri (the ligands only) to establish whether the necessary components exist for the autocrine or paracrine regulation of early mammalian development by IGFs.

Materials and Methods

Collection of embryos and reproductive tracts

Prepubertal Sprague–Dawley rats (26–28 days old) from Charles River Canada (St Constant, Quebec) were induced to ovulate by s.c. injections of 15 iu eCG and, 56 h later, i.p. injections of 15 iu hCG. Ovulated oocytes were collected from oviducts 16–18 h after hCG injections and stripped free of cumulus cells in 0.1% (w/v) hyaluronidase solution in Ca²⁺-, Mg²⁺-free phosphate-buffered saline (PBS). To collect embryos, the rats were paired with fertile males of the same strain during the night following hCG injections. Embryos at one-cell, two-cell, four-cell, morula and blastocyst stages were collected from oviducts and uteri at 24, 48, 72, 98 and 120 h, respectively, after hCG injections. The oocytes and embryos were rinsed extensively in Ca²⁺-, Mg²⁺-free PBS containing 0.3% (w/v) polyvinyl pyrrolidone and frozen at –70°C until further processing.

Oviducts and uteri were collected from untreated, prepubertal rats or prepubertal rats at various times after induction of ovulation and fertile matings as for embryo donors. The

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Table 1. Oligonucleotide primers used for RT-PCR

| Gene | Amplified fragment Predicted restriction | | Primer sequence (5'-3') ^a | Reference |
|----------------------|---|---------------|---|--|
| | Size (bp) | Site | | |
| Insulin receptor | 324 | <i>HincII</i> | 5' Primer GTC <u>ACTG</u> CAGTCATGCCATGTGCTGG (nucleotides 3805-3831) 3' Primer CCGCCCGTT <u>AAG</u> CTTGCCCTCCGTTTCAT (nucleotides 4128-4102) | Ullrich <i>et al.</i> , 1985 |
| IGF Type I receptor | 354 | <i>BanII</i> | 5' Primer ATGCTGTTTGA <u>ACTG</u> CAGCGCATGTGCTGG (nucleotides 3763-3792) 3' Primer CCGCTCGAGCTTGCGGCCCCCGTTTCAT (nucleotides 3116-4090) | Ullrich <i>et al.</i> , 1986 |
| IGF Type II receptor | 186 | <i>HaeIII</i> | 5' Primer TGTACACTCTTCTTCTGGCA (nucleotides 6946-6965) 3' Primer ACAGATGTTGATGTAGAAGACAGG (nucleotides 7140-7177) | Morgan <i>et al.</i> , 1987 ^b |
| IGF-I | 210 | <i>MspI</i> | 5' Primer GGACCGAGACCCTTTGCGGGG (nucleotides 184-205) 3' Primer GGCTGCTTTTGTAGGCTTCAGTGG (nucleotides 393-370) | Bell <i>et al.</i> , 1986 |
| IGF-II | 255 | <i>SacII</i> | 5' Primer CGCCCCAGCGAGACTCTGTGC (nucleotides 1142-1162) 3' Primer GCCCACGGGGTATCTGGGGAA (nucleotides 1396-1376) | Dull <i>et al.</i> , 1984 |

^aUnderscored bases indicate mismatches introduced to generate a sticky end restriction site for cloning of PCR fragment. Nucleotide positions in parentheses indicate the location of the primer sequences relative to published cDNA sequences.

^bSequences modified slightly from human cDNA sequence on the basis of sequences in rat IGF-II receptor cDNA (D. Morgan, unpublished).

oviducts and uteri were trimmed free of fat, snap-frozen in liquid nitrogen and stored at -70°C for further processing.

RNA extraction

Oocytes and embryos were pooled according to the stage of development (about 200 for oocytes, one-cell and two-cell embryos, or 150 for four-cell, morula and blastocyst stage embryos) and lysed in 100 μl of lysis buffer (4 mol guanidinium thiocyanate l^{-1} ; 25 mmol sodium citrate l^{-1} , pH 7; 0.1 mol 2-mercaptoethanol l^{-1} and 0.5% (w/v) Sarkosyl). 20 μg of *Escherichia coli* rRNA (Boehringer-Mannheim Canada, Laval) was added to this mixture, which was then layered on to 100 μl of a cushion containing 5.7 mol CsCl l^{-1} and 0.1 mol EDTA l^{-1} . The sample was centrifuged at 247 000 g for 4 h at 20°C , using a TL-100 tabletop ultracentrifuge and TL-100 rotor (Beckman Canada, Mississauga), as described by Rappolee *et al.* (1989). The resultant RNA pellet was dissolved in 100 μl of 2.5 mol ammonium acetate l^{-1} and precipitated overnight at -20°C after adding 300 μl isopropyl alcohol. The RNA was pelleted by centrifugation at 10 000 g for 20 min, rinsed with 70% (v/v) ethanol and left to air-dry.

For oviducts and uteri, the tissue was first homogenized in the lysis buffer and then layered over CsCl to obtain RNA as for oocytes and embryos.

Reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA from 150-200 oocytes and embryos and 0.5 μg oviductal and uterine RNA were reverse transcribed by incubation at 42°C for 1.5 h in a 20 μl mixture consisting of 50 mmol Tris l^{-1} , pH 8.3, 60 mmol KCl l^{-1} , 10 mmol DTT l^{-1} , 3 mmol MgCl_2 l^{-1} , 20 ng oligo-dT (Pharmacia Canada, Montreal) μl^{-1} , 1 mmol each of dATP, dCTP, dGTP and dTTP l^{-1} (Pharmacia Canada), 2 U RNAase inhibitor (RNasin, Promega, Madison, WI) μl^{-1} , and 1.5 U avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim Canada) μl^{-1} . The reaction was terminated by heating at 90°C for 5 min and quickly cooling in ice.

An aliquot (1.0 μl) of this RT reaction mix was used for PCR in a final volume of 50 μl containing 10 mmol Tris-HCl l^{-1} , pH 8.3, 50 mmol KCl l^{-1} , 1.5 mmol MgCl_2 l^{-1} , 0.001% (w/v) gelatin, 0.2 mmol each of dNTP l^{-1} , 1 μmol each of PCR primers l^{-1} (see below) and 2.5 U Taq DNA polymerase (Gibco-BRL Canada, Burlington). The PCR reaction was covered with 60 μl mineral oil and subjected to 30-40 cycles in a DNA thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) where each amplification cycle consisted of denaturing at 94°C for 1 min, annealing of primers to target sequence at $55-65^{\circ}\text{C}$ for 2 min and primer extension at 72°C for 2 min.

An aliquot (15-20 μl) of the PCR products was analysed by electrophoresis on 7% polyacrylamide or 2% agarose gels and

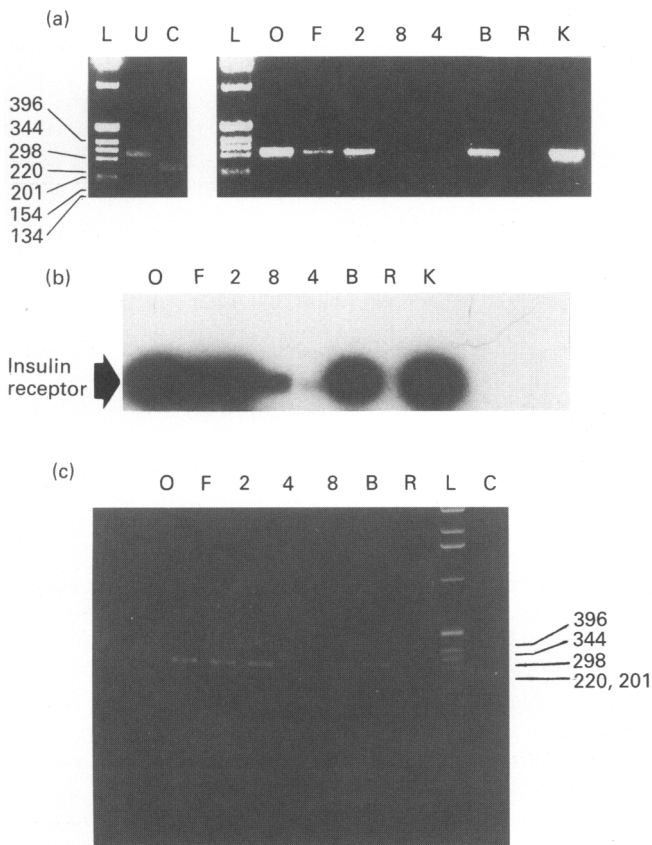


Fig. 1. Insulin receptor mRNA in oocytes and embryos. Approximately 20 oocytes (O), 20 embryos at one-cell (F) or two-cell (2) stages, or 15 embryos at eight-cell (8), four-cell (4) or blastocyst (B) stages were subjected to reverse transcription-polymerase chain reaction (RT-PCR). The medium in which the oocytes or embryos were rinsed before RNA extraction was subjected to the same (RT-PCR) procedures as a negative control (R). A sample of rat kidney cDNA was included as a positive control (K). The values indicate the numbers of base pairs for some of the DNA fragments in a 1 Kb DNA ladder (L). (a) Electrophoresis and ethidium bromide staining of PCR products after a 30-cycle amplification. The amplification signal was verified by digestion with *HincII* to yield expected fragment sizes of 255 and 69 bp (the latter fragment was not clearly visible in the gel, probably because of its small size); an example of the digestion of a PCR product from a one-cell embryo (F) is shown on the left (U: uncut; C: cut). (b) Southern blot analysis of the gel in (a) using a ^{32}P -labelled cDNA probe for the human insulin receptor. (c) Electrophoresis and ethidium bromide staining of PCR products after a 40-cycle amplification.

stained with ethidium bromide to visualize PCR products on a UV transilluminator. The authenticity of the PCR products with the anticipated sizes was further verified by either digestion with restriction endonucleases specific for sequences bracketed by the PCR primers, or Southern blot analysis after the PCR products were transferred from an electrophoresis gel to a nylon membrane, as described by Telford *et al.* (1990a) and Watson *et al.* (1992).

The PCR primers used in the present study were designed and synthesized as reported by Telford *et al.* (1990a) and

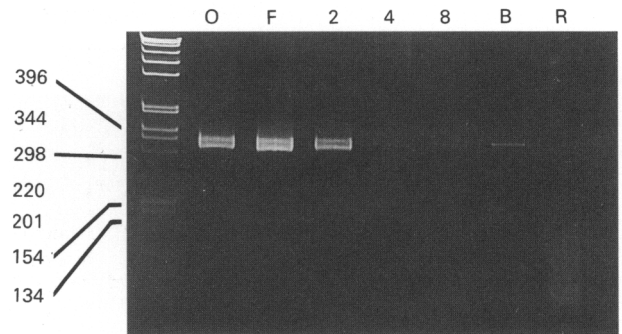


Fig. 2. Insulin-like growth factor (IGF) type I receptor mRNA in oocytes and embryos. A 30-cycle amplification was performed on samples equivalent to approximately 20 oocytes (O), 20 embryos at one-cell (F) or two-cell (2) stages, or 15 embryos at four-cell (4), eight-cell (8) or blastocyst (B) stages. The medium in which the oocytes or embryos were rinsed before RNA extraction was subjected to the same reverse transcription-polymerase chain reaction (RT-PCR) procedures as a negative control (R).

Watson *et al.* (1992). The nucleotide sequences of these primers are shown in Table 1.

Results

All RNA preparations were evaluated by first determining the presence of a relatively abundant mRNA, β -actin mRNA, before the gene of interest was studied. An aliquot of each RNA preparation was also subjected to the RT-PCR procedure, with the omission of reverse transcriptase during the RT reaction. No amplification product was detected in such samples using the PCR primers selected for this study (results not shown). This finding indicates that the amplified signal in our cDNA preparations was not due to the presence of genomic DNA.

After an amplification of 30 cycles, a PCR product corresponding to the insulin receptor mRNA was visualized by ethidium bromide staining in oocytes, one-cell and two-cell embryos and blastocysts (Fig. 1a). Southern blot analysis using a radiolabelled cDNA probe for human insulin receptor (Ullrich *et al.*, 1985) confirmed the authenticity of the PCR product, and also detected insulin receptor mRNA signals in four-cell and eight-cell embryos (Fig. 1b). The presence of low amounts of insulin receptor mRNA in four-cell and eight-cell embryos was further demonstrated when the PCR was continued for 40 cycles (Fig. 1c).

Messenger RNA encoding the IGF type I receptor was also detected in oocytes, one-cell and two-cell embryos and blastocysts (Fig. 2). The signals in four- and eight-cell embryos were too weak to be visualized easily. This was not improved by increasing the number of PCR cycles, due to the presence of a non-specific band (the lower band). This non-specific band has also been observed in other studies on mouse and bovine embryos using the same PCR primers (Telford *et al.*, 1990a; Watson *et al.*, 1992), and is caused by a crossreaction between the primers and insulin receptor cDNA sequences (Telford *et al.*, 1990a). Differential digestion with

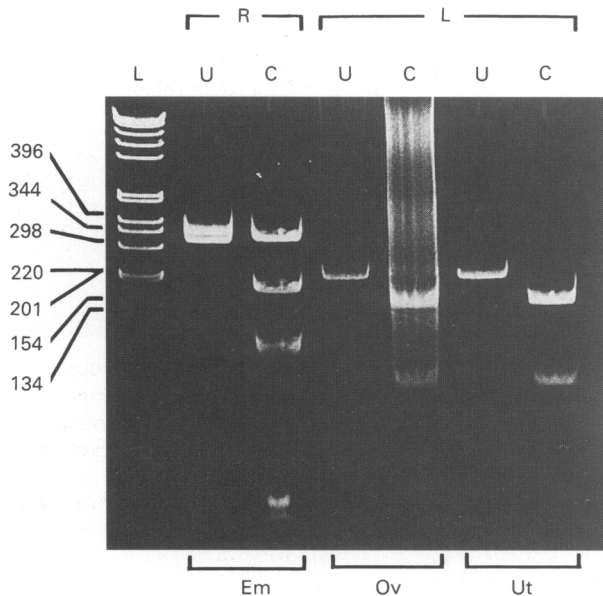


Fig. 3. Verification of polymerase chain reaction (PCR) products. *Ban*II and *Msp*I were used to digest PCR products corresponding to insulin-like growth factor (IGF) type I receptor mRNA (R, 354 bp) and IGF-I mRNA (L, 210 bp), respectively. L: 1 kb ladder; U: uncut; C: cut; Em: embryo (Lane O in Fig. 2); Ov: oviduct (Lane T1 in Fig. 4); Ut: uterus (Lane U1 in Fig. 4). The fragments in the C lane for the IGF-I receptor represent the uncut insulin-receptor PCR product (324 bp) and fragments from *Ban*II digestion of the IGF-I receptor PCR products.

*Ban*II (Fig. 3) verified that the upper band (354 bp) represents the amplification product corresponding to the mRNA encoding the IGF type I receptor.

IGF-I mRNA was not detected in oocytes or preimplantation embryos after a 40-cycle amplification. This was not due to the failure of the experimental procedures, because in each experiment a rat kidney sample, included as a control, always yielded a PCR product corresponding to IGF-I mRNA. Furthermore,

IGF-I mRNA was detected in oviducts and uteri (Fig. 4), and the authenticity of the PCR product was confirmed by digestion with *Msp*I (Fig. 3).

IGF type II receptor mRNA was present in oocytes and embryos of all stages, and digestion with *Hae*III produced diagnostic fragments of the expected size (Fig. 5). IGF-II mRNA (Fig. 6) was present in oocytes and embryos of all the stages tested. The PCR product was verified by digestion with *Sac*II (Fig. 6).

Discussion

Insulin and IGFs are a group of structurally related polypeptides that regulate the growth of many types of mammalian cell (reviewed by Froesch *et al.*, 1985; and Czech, 1989). The growth-promoting actions of these polypeptides are mediated by binding to receptors on the cell membrane, and receptors with primary binding specificity for the polypeptides have been identified (reviewed by Baxter, 1988). IGF-I has a higher binding affinity for the type I receptor than for the type II receptor, and IGF-II has a higher binding affinity for the type II receptor than for the type I receptor. Insulin can also interact with the type I receptor. The present study demonstrates the presence of mRNA encoding the insulin receptor in preimplantation rat embryos, and provides supporting evidence that the previously observed beneficial effect of insulin on rat embryo development could be mediated through its specific receptor. The genes encoding IGF type I and type II receptors are also expressed in rat oocytes and preimplantation embryos at some (for type I receptor) or all stages examined. This suggests that, as in mice (Dechiara *et al.*, 1990; Harvey and Kaye, 1988), IGFs also play a role in normal preimplantation development in the rat by interacting with their specific receptors in the embryo.

IGFs are produced by many tissues of the body and therefore can act in a paracrine or autocrine fashion in addition to the endocrine mode of action that is used by insulin (D'Ercole *et al.*, 1984). Thus, while bloodborne insulin may transude across the reproductive tract to exert its action on rat

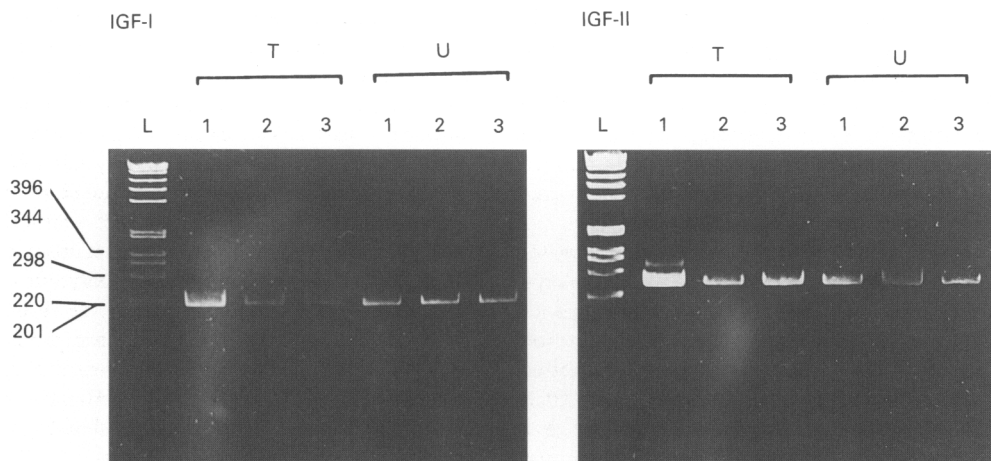


Fig. 4. Insulin-like growth factor (IGF) type I and II mRNA in oviducts and uteri. A 30-cycle amplification was performed on samples of oviducts (T) or uteri (U) from untreated, prepubertal rats (1), or rats on day 2 (2) or day 5 (3) of pregnancy (day 1: presence of copulatory plug or spermatozoa in vagina). L: 1 kb ladder.

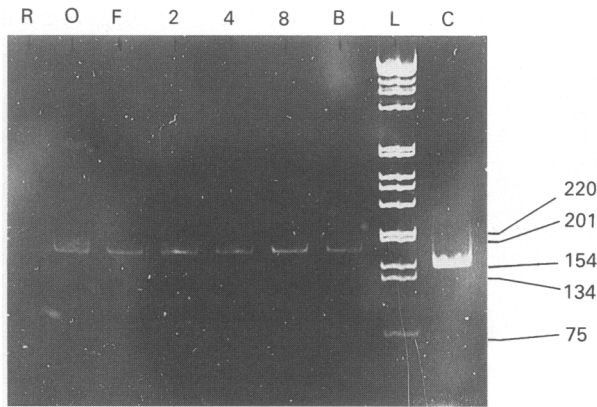


Fig. 5. Insulin-like growth factor (IGF) type II receptor mRNA in oocytes and embryos. A 30-cycle amplification was performed on samples equivalent to approximately 20 oocytes (O), 20 embryos at one-cell (F) or two-cell (2) stages, or 15 embryos at four-cell (4), eight-cell (8) or blastocyst (B) stages. The medium in which the oocytes or embryos were rinsed before RNA extraction was subjected to the same reverse transcription–polymerase chain reaction (RT–PCR) procedures as a negative control (R). The PCR products were verified by digestion with *Hae*III to yield the expected 152 and 34 bp fragments (the latter fragment was not clearly visible in the gel, probably because of its small size); an example of the digestion of a PCR product from a one-cell embryo (F) is shown to the right of the 1 kb ladder (C). L: 1 kb ladder.

embryos, IGFs that are destined to interact with embryos may come from the general blood circulation, the reproductive tract or embryos. The present study did not examine whether insulin mRNA is present in embryos or the reproductive tract. However, insulin mRNA was not detected in preimplantation embryos from mice (Rappolee *et al.*, 1990). IGF-I mRNA could not be detected in preimplantation embryos in the present study, which is consistent with the absence of this mRNA in preimplantation mouse embryos (Rappolee *et al.*, 1990). This indicates that an autocrine mechanism is not the likely mode of action for this polypeptide during preimplantation development. The oviduct and uterus are possible sources for IGF-I, since its mRNA is detected in these two tissues. For IGF-II, both autocrine and paracrine interactions may mediate its action since its mRNA is present in preimplantation embryos and the reproductive tract. The present study did not examine whether the IGF mRNAs are translated in the reproductive tract. If these IGF polypeptides are present, their actions on embryo development would be further regulated by various IGF binding proteins, which have yet to be studied in this context.

Preimplantation development of embryos from many mammalian species can be maintained *in vitro*, independent of the maternal environment. However, the rate of embryo development *in vitro* is impaired to various degrees depending on the species and can be improved by co-culture with oviductal or uterine cells as well as with some other types of cell (Bavister, 1988). This finding suggests that mammalian embryos have certain autonomy in regulating their early development, but that optimal development is obtained by support from their physiological environment. This hypothesis for the control of early mammalian development is well supported by the present

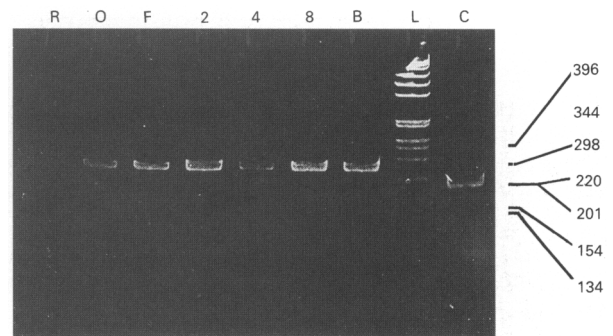


Fig. 6. Insulin-like growth factor (IGF) type II mRNA in oocytes and embryos. A 30-cycle amplification was performed on samples equivalent to approximately 20 oocytes (O), 20 embryos at one-cell (F) or 2-cell (2) stages, or 15 embryos at 4-cell (4), 8-cell (8) or blastocyst (B) stages. The medium in which the oocytes or embryos were rinsed before RNA extraction was subjected to the same reverse transcription–polymerase chain reaction (RT–PCR) procedures as a negative control (R). The PCR products were verified by digestion with *Sac*II to yield expected fragments of 189 and 67 bp; an example of the digestion of a PCR product from a one-cell embryo (F) is shown to the right of the 1 kb ladder (C). L: 1 kb ladder.

data on gene expression for the receptors of insulin and IGFs as well as IGF ligands. Thus, while IGF-II may be a component of autonomous regulation, IGF-I and insulin are very likely to be some of the possible maternal regulators of preimplantation development. Further evidence that insulin plays an important role in early development comes from experiments in which mice or rats have been made insulin-deficient through streptozotocin treatment. Blastocysts collected from such diabetic mothers have fewer cells than do their normal counterparts (Pampfer *et al.*, 1990; Beebe and Kaye, 1991).

Some of the earliest mammalian developmental events are controlled by maternal products accumulated within the oocyte during oogenesis. These inherited maternal transcripts undergo a degradation process to be replaced by transcripts of the embryonic genome at various species-specific stages of early cleavage (Telford *et al.*, 1990b). In mouse embryos, the transition from maternal to embryonic genome control occurs after the first cell division, and this transition is often reflected in the changing levels of many mRNA species at the two-cell stage. For example, the amount of β -actin mRNA decreases at the early two-cell stage, as a result of the breakdown of maternal transcripts in embryos, and increases thereafter as a result of the activation of the embryonic genome (Giebelhaus *et al.*, 1983; Graves *et al.*, 1985). In the study by Rappolee *et al.* (1990), none of the genes that were examined in the present study was expressed in mouse embryos before the two-cell stage (i.e. before activation of the embryonic genome), suggesting that maternal transcripts of these genes are not inherited by mouse embryos. The results from the present study suggest that this is not the case for the early rat embryo since maternal messages for some of the growth factors (i.e. mRNAs encoding IGF-II and receptors for insulin and IGFs) were observed in unfertilized rat oocytes and embryos.

Similar differences in the patterns of gene expression were also reported between bovine embryos and mouse embryos (Watson *et al.*, 1992), suggesting that growth factors may exert

an influence on preimplantation development in many mammalian species earlier than that reported in mice. Although no definitive evidence is available for the time of activation of the embryonic genome in rats, it appears to occur after the two-cell stage as in mice. The low concentration of mRNA encoding the insulin receptor and IGF type I receptor in four-cell and eight-cell embryos may be due to a delay in activation of these genes in the embryonic genome.

This study takes advantage of the highly sensitive RT-PCR procedure to analyse gene expression in early mammalian embryos that often cannot be analysed biochemically because of the scarcity of experimental material. Further studies are required to verify the translational status of the mRNAs that are studied in this report. This will help confirm the physiological significance of insulin and IGFs in the regulation of early embryo development.

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