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Original article

Comparative expression of TGF-α and EGF genes in the ovine conceptus and uterine endometrium in the peri-implantation period

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Summary — Control of growth and differentiation during mammalian embryogenesis may be regulated by growth factors. With the use of a very sensitive method, the reverse transcription-polymerase chain reaction, expression of some growth factor transcripts was examined during ovine peri-implantation development. Transcripts for transforming growth factor-α (TGF-α) were found in day 15 to day 30 conceptuses, and in uterine tissues. Epidermal growth factor (EGF) was not detectable in any ovine stages or tissues studied. TGF-α could be the normal physiological ligand for the epidermal growth factor receptor present on the trophoblastic tissues, and its expression pattern suggests an autocrine and paracrine role in the growth and differentiation of ovine embryos.

epidermal growth factor / transforming growth factor alpha / gene expression / sheep embryo / uterine endometrium

Résumé — Étude comparée de l'expression des gènes du TGF-α et de l'EGF par le conceptus ovin et l'endomètre utérin en période péri-implantatoire. *Chez les Mammifères, la croissance et la différenciation des embryons sont régulées par des facteurs de croissance. À l'aide d'une méthode très sensible, la transcriptase inverse suivie de réaction de polymérisation en chaîne (RT-PCR), l'expression de l'EGF et du TGF-α, a été examinée au cours du développement embryonnaire péri-implantatoire de l'Ovin. Des transcrits du TGF-α sont observés sur des conceptus âgés de 15 à 30 j ainsi qu'au niveau de l'endomètre utérin prélevé entre le 5e et le 30e jour de gestation. Quel que soit le stade considéré, l'EGF n'est détecté dans aucun des tissus étudiés. Le TGF-α pourrait être le ligand naturel, se fixant au récepteur trophoblastique de l'EGF. Son expression par le conceptus (embryon et trophoblaste) et l'endomètre suggère un contrôle à la fois autocrine et paracrine sur le développement.*

epidermal growth factor / transforming growth factor alpha / expression des gènes / embryon ovin / endomètre utérin

INTRODUCTION

Development is a series of processes that includes cell proliferation, differentiation, migration and invasion. One set of molecules that seems to have a central role in regulating all of these processes is the polypeptide growth factors. The presence of functional epidermal growth factor receptors (EGF-R) in ovine trophoblastic tissues (Gharib-Hamrouche *et al,* 1993) suggests that epidermal growth factor (EGF) or EGF-related proteins could be involved in embryonic and fetal development. The role of EGF in morphogenetic events and differentiation has been studied in several animal species. EGF stimulates proliferation of embryonic cells in culture (Yoneda and Pratt, 1981; Kaplowitz *et al,* 1982) and during morphogenesis (Goldin and Opperman, 1980; Pratt, 1980). The transforming growth factor-α (TGF-α), which is an EGF-related growth promoting polypeptide, binds to and interacts through the EGF-receptor (Massagué, 1983; Downward *et al,* 1984). It was first discovered from culture of murine sarcoma virus-transformed cells and was later isolated from culture of various tumor cells (DeLarco and Todaro, 1978; Todaro *et al,* 1980). Recently, TGF-α has been demonstrated in normal adult tissues, such as bovine pituitary (Samsoondar *et al,* 1986), normal epithelial skin keratinocytes (Coffey *et al,* 1987), regenerating rat liver (Mead and Fausto, 1989), and brain (Kudlow *et al,* 1989). Since TGF-α, but not EGF, is also found in the preimplantation mouse embryo and in several embryonic midgestational mouse tissues (Rappolee *et al,* 1988a, Wilcox and Derynck, 1988), it is possible that TGF-α represents a fetal analog of EGF, which is normally expressed in stem cells and becomes inappropriately overexpressed in transforming cells (Derynck, 1988).

The recently described technique of reverse transcription-polymerase chain reaction (RT-PCR) provides a very sensitive amplification method for demonstrating the presence of mRNA in small volumes of tissue and/or when the mRNA is present in very low copy number. In this study, we have applied the RT-PCR to determine whether mRNA for EGF and TGF-α were present in ovine conceptus during the peri-implantation period.

MATERIALS AND METHODS

Animals and tissues

Oestrus was synchronized in Préalpes du sud ewes with intravaginal sponges (40 mg, 17α-acetoxy-9α-fluoro-11β-hydroxyprogesterone; Intervet, France), which were removed after 14 d. Ewes then received one im injection of 500 iu PMSG (Intervet, France). On the day of oestrus, which occurred 48 h later and was designated as day 0, ewes were mated twice. Ewes were slaughtered on days 5, 15, 17, 20, 25 and 30 of gestation and the uterus was removed. On day 15 of pregnancy, embryos were collected by flushing the uteri with sterile phosphate-buffered saline (PBS) 0.1 mol•l^{-1}, pH 7.2; from days 20 to 30 of pregnancy, conceptuses were recovered by incising the uterine horns. Embryos and trophoblasts could be easily separated at this period. Tissue endometrium was obtained from the uterine cavity at 5, 15, 17, 20, 25 and 30 d of gestation.

Immediately after collection, tissue samples were quickly flash-frozen in liquid nitrogen and stored at –80°C until required.

RNA preparation

Total RNA extraction was performed with the original method using acid guanidinium thiocyanate/phenol/chloroform (Chomczynski and Sacchi, 1987) slightly modified by Puissant and Houdebine (1990). RNAs were quantified by UV absorption measurements, precipitated by ethanol and stored at –20°C until further analyses.

cDNA synthesis

RNA from embryos, trophoblasts and endometrium were reverse transcribed into cDNA (Gubler and Hoffman, 1983). In brief, purified RNA equivalent to 1 μg, was incubated at 42°C for 60 min with a mixture of 200 U of M-MuLV reverse transcriptase (obtained from BRL Gibco, USA) and the following reagents (final volume 20 μl): 10 mM dithiotreitol; 3 mM $MgCl_2$; 10 mM Tris-HCl buffer, pH 8.4; 0.5 μg oligo-dT primer (12-18 mers; Pharmacia, France); 2 μg nuclease-free bovine serum albumin; 0.5 mM dNTP (ATP, TTP, CTP, GTP; Pharmacia, France); and 5 U of RNAse inhibitor (Boehringer Mannhein Biochemica, France). The reverse transcriptase enzyme was inactivated by heating at 93°C for 5 min before using the cDNA.

Oligonucleotide design

Primer pairs were obtained from Bioprobe systems (France). The nucleotide sequences synthesized to anneal with cDNA and the sizes of the expected PCR fragment are shown in table I. A primer pair for mouse β-actin cDNA was included as an internal control. Cytoplasmic β-actin transcripts are expected to be present in all tissues. This primer pair can therefore be used both to detect the presence of β-actin mRNA (cDNA) *via* a predicted 243 bp PCR fragment and to fully check mRNA.

Polymerase chain reaction

PCR was performed essentially as previously described by Saiki *et al* (1988). Aliquots of the RT reaction (2–3 μl) were amplified with 1 U of Taq-polymerase (Bioprobe systems, France) in a final volume of 50 μl containing 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 5 μg nuclease free BSA, 1 μM of each sequence specific primer, and 0.2 mM dNTP. The mixture was overlaid with mineral oil to prevent evaporation and then amplified by PCR for 50 cycles in a DNA thermocycler programmable heating block (Trio-thermoblock TB-1, Biometra, Germany) where each cycle included denaturation at 94°C for 1 min, annealing of primers to target sequences at 55°C for 2 min, and primer extension at 72°C for 2 min. The final cycle included polymerization for 7 min for complete strand extension.

Agarose gel electrophoresis

A portion of the PCR products (5 μl) was added to 2 μl of loading dye mix and electrophoresed in a 80 V constant-voltage field in a 2% agarose gel containing 0.5 $mg \cdot ml^{-1}$ ethidium bromide and photographed.

Table I. Oligonucleotide sequences of the primers used to perform the PCR to identify cDNA for β-actin, EGF, and TGF-α.

Gene	*Size (bp)*	*Primer sequence* [a]	*Reference*
β-Actin	243	-CGTGGGCCGCCCTAGGCACCA-(182–202) -TTGGCCTTAGGGTTCAGGGGGG-(424–403)	Tokunaga *et al* (1986)
EGF	247	-CCAGTTCAGTAGAAATCGGG-(3 953–3 972) -TGGTTTCTAATGATTTTCTC-(4 200–4 181)	Gray *et al* (1983)
TGF-α	175	-ACCTGCAGGTTTTTGGTGCAG-(232–252) -GGACCGACAGGAATAGATAGT-(387–406)	Derynck *et al* (1986)

[a] Numbers in parentheses following the primer sequence indicate the nucleotide sequence location of the primer in the published cDNA or genomic sequence.

Hybridization cDNA probe

The human TGF-α cDNA clone ph TGF1 10-925 / HB 101 / Amp ^{R} / vector pB 327/925 bp Eco-R1 insert was kindly provided by Dr GI Bell (Howard Hughes Medical Institute, University of Chicago, USA). The plasmid pAL 41 contains the coding sequence of the mouse β-cytoplasmic actin (Alonso *et al*, 1986). This was a gift of Dr M Buckingham (Institut d'Embryologie, Nogent-sur-Marne, France). Inserts were labelled with α^{32}P d-CTP (3000 Ci mmol^{-1}; Amersham, France) using 'Klenow fragment' (Boehringer Mannheim Biochemica, France) to specific activity of 1.5 x 10^{9} cpm•μg^{-1} according to the method of Feinberg and Vogelstein (1984). The probe was purified by filtration on quick spin TM columns G25 Sephadex (Boehringer Mannheim Biochemica, France).

DNA blot analysis

In order to confirm the identity of the PCR products, DNA bands separated on agarose gel were transferred to Zeta-Probe nylon membrane (Bio Rad, USA) as described by Reed and Mann (1985) and probed with the appropriate sequence according to Maniatis *et al* (1982) by the following protocol: gels were treated at ambient temperature, successively with HCl 0.25 M for 10 min, NaOH 0.5 M for 30 min and Tris-HCl 0.5 M NaCl 3 M, pH 7.4 for 30 min. The gels were then incubated in contact with nylon membrane overnight in 10 SSC (1 SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Zeta-bind transfers were rinsed in 1SSC, hybridized with radiolabelled probe and washed at 60°C for 60 min in 0.2 SSC containing 0.1% sodium dodecyl sulfate before exposure to hyperfilm B_{max} (Amersham International plc) for 10 or 15 min at –80°C, with intensifying screens.

RESULTS

The sensitive technique of mRNA phenotyping by RT-PCR was applied to total RNA samples. Because of the small amount of embryonic material that we could get at day 15 of pregnancy, total RNA was isolated from pooled embryonic discs as well as trophoblastic tissues. At the post-implantation stages of gestation, *ie* days 20, 25 and 30, single embryos were separated from trophoblasts and both were used for the preparation of total RNA. In this manner, we have searched for detectable levels of transcripts encoding EGF and TGF-α in peri-implantation embryos, trophoblasts and uterine endometrium. For each stage considered and tissue studied, the assays were repeated at least 3 times with different RNA preparations. The purified RNA was primed with oligo-dT and reverse transcribed and then aliquots of the product were used for amplification by PCR for each specific primer pair. All the preparations were tested for the presence of contaminating genomic DNA by amplifying with actin primers. No PCR product for actin genomic DNA was detected, whatever the samples, without prior RT assay. Genomic actin sequences give rise to a PCR DNA band larger (approximately 400 bp) than the expected 243 bp band, which is derived from β-actin mRNA (cDNA). Without reverse transcription (-RT), no PCR product can be obtained in any of the β-actin or growth factor assays (fig 1, 3, 4, 5). Examples of PCR results that display the typical pattern of expression are presented in this study. Transcripts for β-actin were detected within all of the embryo stages (fig 1A) and uterine tissue (fig 1B). The identity of the expected 243 bp PCR fragment was confirmed by Southern blot analysis (fig 2).

The most significant result of the PCR was to obtain a dominant TGF-α product of the predicted length (175 bp). From these results, it was apparent that TGF-α was expressed in the embryo (fig 3A) and the trophoblast (fig 3B). The factor was present at the day 15 of gestation and remains until the day 30. TGF-α mRNA were also expressed by gestational uterine tissues from days 5, 15 and 20 (fig 4), but no difference was observed between caroncular and intercaroncular tissue at the implantation (fig 5).

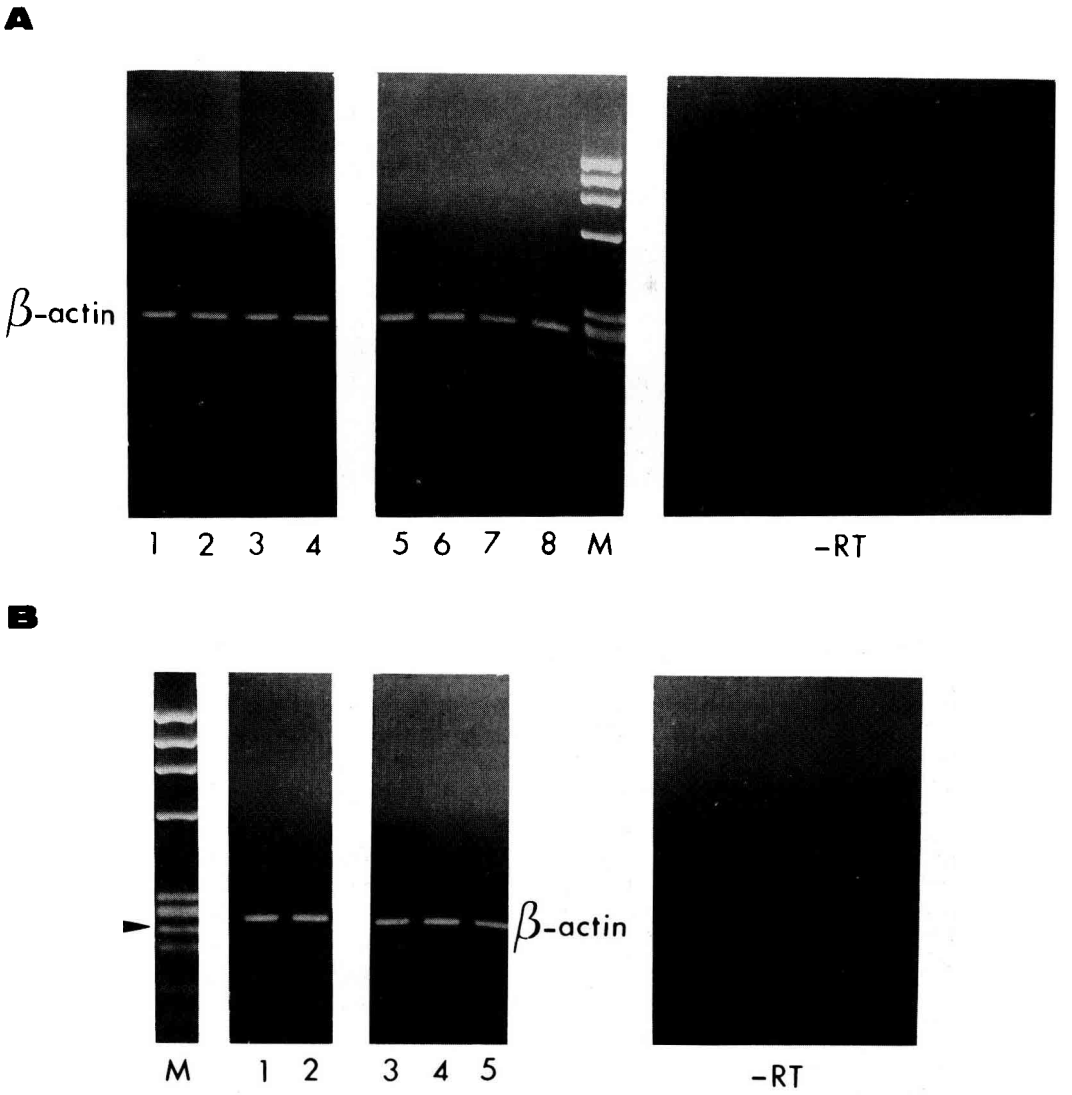

Fig 1. Expression of β-actin transcripts detected by RT-PCR in RNA isolated from ovine samples. The amplified DNA fragments were electrophoresed through a 2% agarose gel with 2 μg of 1 kb ladder (lanes marked **M**), stained with ethidium bromide, and viewed over an ultraviolet transluminator. Each lane numbered represents the β-actin PCR products from (**A**) peri-implantation ovine conceptus disposed as follows: **1**) day 15 embryo; **2**) day 20 embryo; **3**) day 25 embryo; **4**) day 30 embryo; **5**) day 15 trophoblast; **6**) day 20 trophoblast; **7**) day 25 trophoblast; **8**) day 30 trophoblast; and (**B**) uterine endometrium from pre-implantation (day 5, lane **1**), implantation (day 15, lane **2**), and post-implantation stages (days 20, 25, and 30 of gestation, respectively, in lanes **3**, **4** and **5**). No contaminating genomic DNA was observed because without reverse transcription (**-RT**) any product can be obtained by PCR.

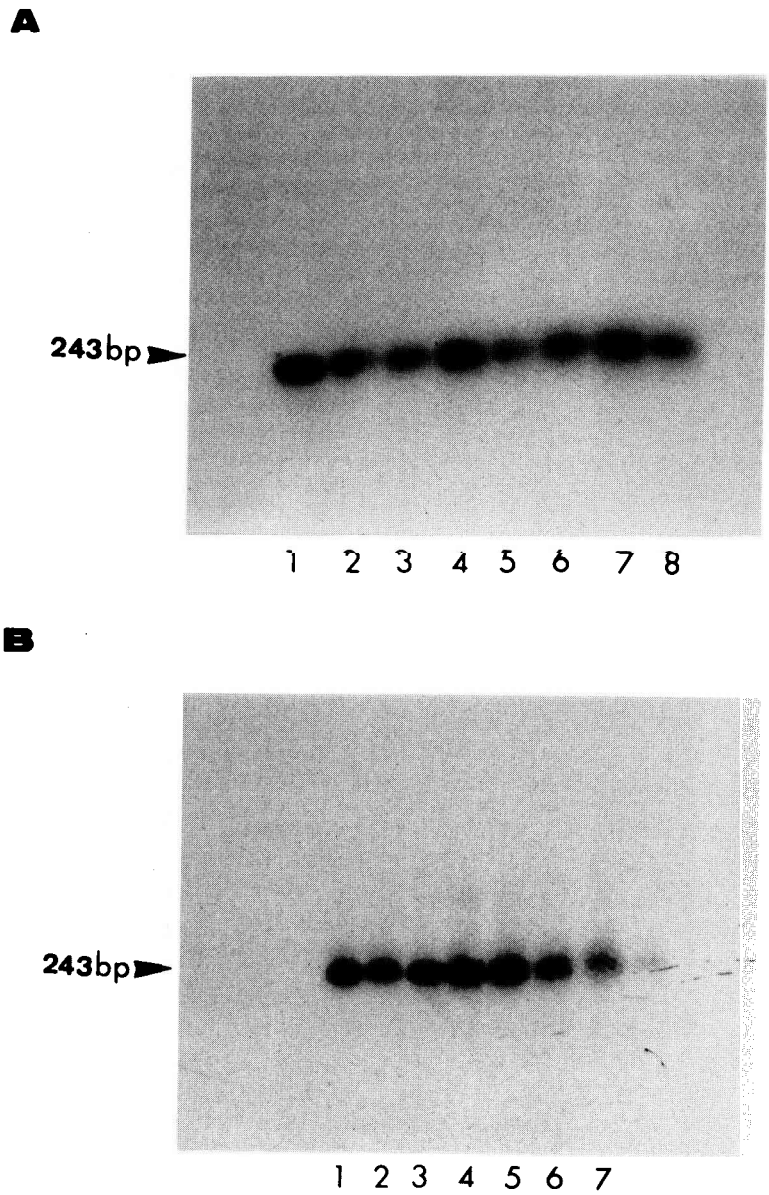

Fig 2. Autoradiogram of Southern blot analysis of cDNA hybridized with β-actin probe to confirm that the 243 bp amplified DNA fragment (arrow) was representative of β-actin transcripts from (**A**) ovine conceptus, lanes **1–4** (embryo from day 15–30 of gestation), and lanes **5–8** (the corresponding trophoblast) and (**B**) uterine endometrium from day 5–30 of pregnancy (lanes **1–5**), with a comparison between caroncular (lane **6**) and intercaruncular (lane **7**) tissue from day 17.

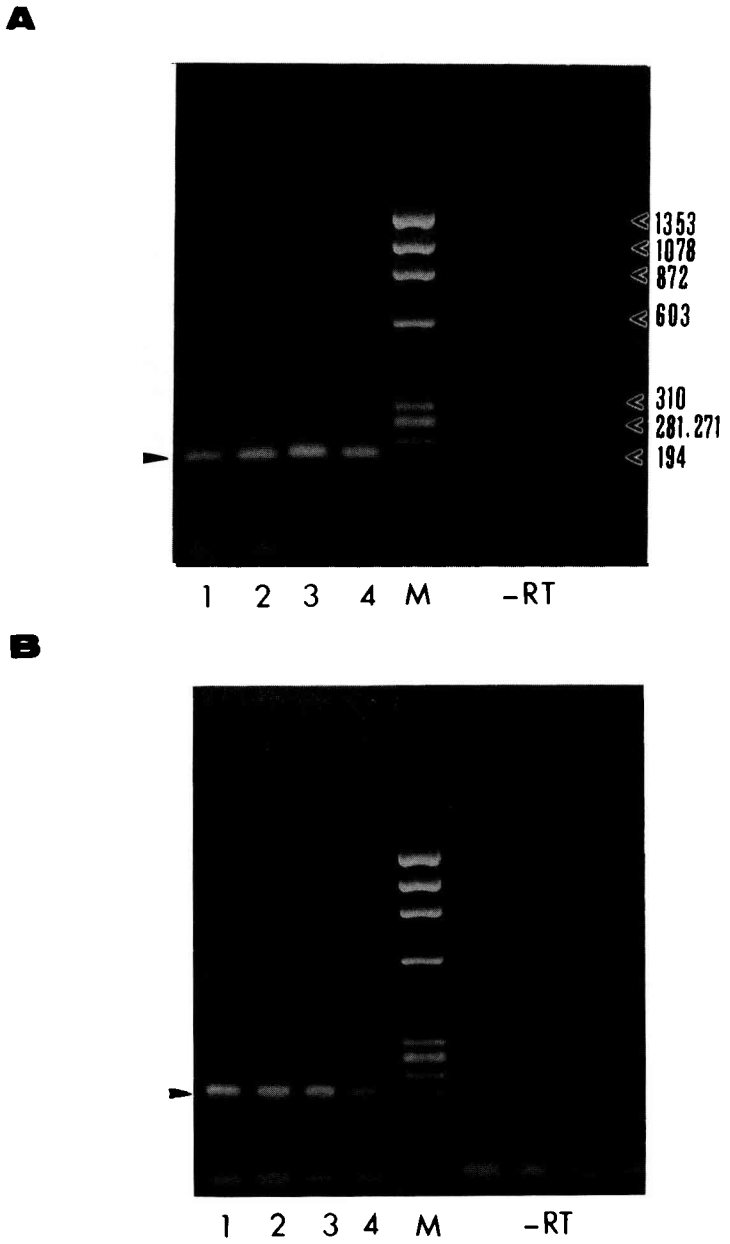

Fig 3. Detection of mRNA transcripts for transforming growth factor-α (TGF-α) in ovine conceptus, produced using a cDNA aliquot derived from (**A**) embryos and (**B**) trophoblast, resolved on 2% agarose gel with ladder molecular weight (lane **M**). The PCR products are disposed as follows: **1**) day 15; **2**) day 20; **3**) day 25; **4**) day 30 of pregnancy. The sizes of Hae III digested Φx 174-RF DNA size marker (**M**) are indicated in base pairs on the right. Without reverse transcription (**-RT**), no PCR fragment can be observed.

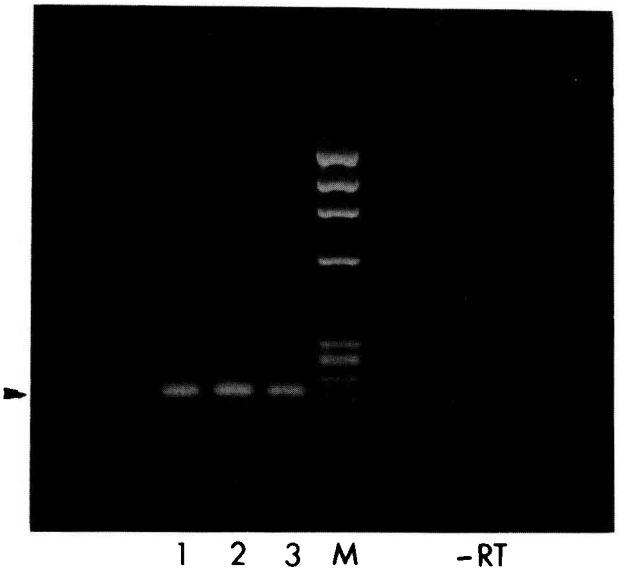

Fig 4. Detection of TGF-α transcripts (**arrow**) within ovine peri-implantation uterine endometrium. The PCR products from days 5 (lane **1**), 15 (lane **2**), 20 (lane **3**) of gestation were resolved on a 2% agarose gel with 2 μg of 1 kb DNA ladder (lane **M**). The lanes on right represent the same PCR uterine samples without reverse transcription (**-RT**).

The PCR bands were verified as a TGF-α target fragment by blotting and hybridizing with a partial human TGF-α cDNA probe. The autoradiogram band corresponded exactly to the PCR product confirming the identity of the growth factor (fig 6).

In contrast, EGF expression was not detected in any of the ovine embryonic, trophoblastic, and uterine RT-PCR assays (fig 7), although a strong EGF band (247 bp) was observed in the positive control using cDNA derived from human salivary gland total RNA (Clontech, Laboratories Inc, USA).

DISCUSSION

Peptide growth factors and their receptors play an important role in the development of various organisms. This study demonstrates the power of RT-PCR in identifying the presence of mRNA in peptides in which the message is present in low copy number. Unlike Northern blot hybridization and dot blots, it does not require large amounts of tissue, because there is no need for the preparation of poly (A^+) RNA.

In our findings, cDNA contamination was unlikely because amplification of genomic DNA was prevented by PCR by using the primers in the absence of template DNA, and failed to show a product.

The oligonucleotide primers used for PCR were designed using mouse or human cDNA sequences. At the present time, TGF-α DNA sequences of human (Derynck *et al*, 1984) and rat (Lee *et al*, 1985) are known. They show 94% sequence homology in the coding sequence for the mature TGF-α peptide. Therefore, there is good reason to

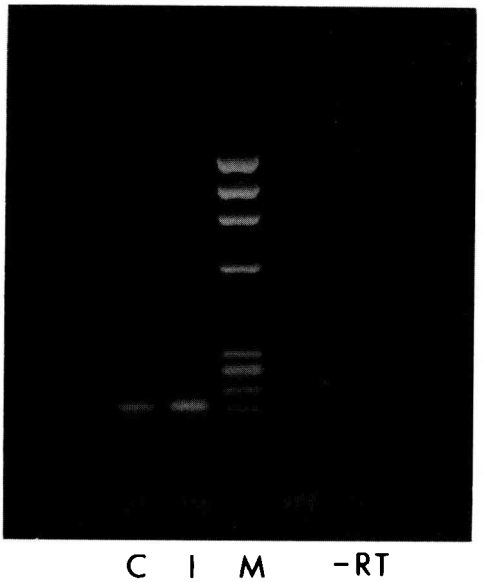

Fig 5. Detection of TGF-α transcripts (arrow) within implantation (day 17) uterine endometrium. The 175 bp PCR fragment was present in caroncular (**C**) and intercaroncular (**I**) tissue samples, running on agarose gel (2%) with 2 μg of ladder marker sizes (lane **M**). If reverse transcription was not applied (**-RT**), no PCR product could be detected.

believe that the coding sequence for the mature TGF-α peptide is highly conserved. The primers' efficacy with ovine cDNA was determined by ascertaining that the resulting fragment was of the expected size, and then could blot and hybridize with the appropriate human cDNA probe.

With the assistance of PCR, we have identified TGF-α expressed by ovine embryo and trophoblast at the implantation stage (day 15). This expression still remains after, for example, days 20, 25 and 30 of pregnancy. RT-PCR has previously been used to demonstrate mRNA for TGF-α in mouse fetal tissue (Rappolee *et al,* 1988a, b), and thereafter Werb (1990) showed that this growth factor was expressed as early as the 4-cell developmental stage by mice embryos. In this study, we cannot precise the stage at which the transcripts are first detectable in sheep, because the great number of blastocysts needed for early investigations. However, Watson *et al* (1994) recently demonstrated the expression of TGF-α throughout ovine preimplantation development (from the 1-cell zygote to the early cavitation or fully expanded blastocyst). On the other hand, we have established that endometrium at the peri-implantation stages (from day 5 to day 25 of pregnancy) is a source of TGF-α, even if it is not possible to determine whether the uterine epithelial or stromal cells are involved.

Transcripts for EGF were not detected at any developmental stage of the ovine peri-implantation embryo and trophoblast or within the uterine endometrium preparations, according to previous bovine and ovine preimplantation investigations (Watson *et al,* 1992, 1994). However, the expected 247 bp band was not only observed with cDNA derived from human salivary gland total RNA (our study), but also in a homologous system using ovine ovary RNA preparation (Watson *et al,* 1992).

The detection of TGF-α mRNA supposes but does not prove peptide secretion by the tissues examined. It is well known that TGF-α is a cleavage product of a 160

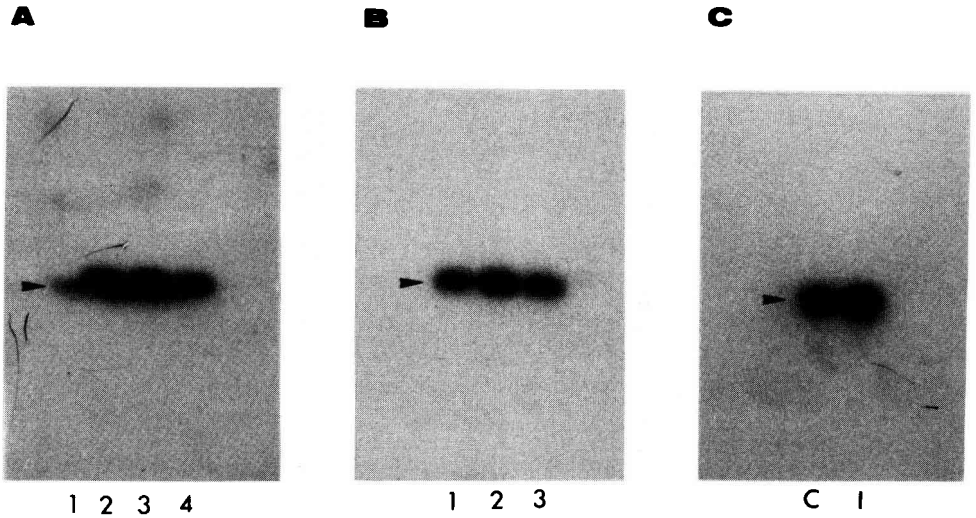

Fig 6. Verification of TGF-α RT-PCR products. PCR fragments for identification purposes were produced from cDNA preparations of peri-implantation ovine tissues: (**A**) conceptus from days 15, 20, 25 and 30 of pregnancy (respectively lanes 1, 2, 3 and 4); (**B**) uterine endometrium from days 5, 15 and 20 of gestation (lanes 1, 2 and 3); and (**C**) caroncular (C) and intercaroncular (I) comparison. After blotting and hybridization with a partial human TGF-α probe, the autoradiogram shows that the radiolabelled probe hybridizes specificially to the 175 bp PCR fragments (arrow).

amino-acid transmembrane polypeptide: the pro-TGF-α (Derynck *et al,* 1984; Lee *et al,* 1985). The TGF-α sequence starts with residue 40 in the extracellular domain of pro-TGF-α, but the proteolytic process that releases mature TGF-α from pro-TGF-α is inefficient in most cell types. Consequently, substantial levels of pro-TGF-α can accumulate on the cell surface. The possibility that membrane pro-TGF-α might be biologically active while anchored on the cell surface has been demonstrated by using genes mutated *in vitro* to encode non-cleavable forms of pro-TGF-α (Brachmann *et al,* 1989; Wong *et al,* 1989). Pro-TGF-α can bind to EGF/TGF-α receptors located on the surface adjacent cells and activate the receptor tyrosine kinase autophosphorylation. Furthermore, this interaction leads to a rapid rise in cytosolic calcium levels, a response typical of signalling by EGF receptors (Carpenter and Cohen, 1979), even if non-cleavable pro-TGF-α is less potent than soluble TGF-α as an activator of EGF receptors. Given its high affinity for the EGF receptor, membrane pro-TGF-α may act as a mediator of cell–cell homing and adhesion. This function has been demonstrated by co-culturing non-adherent EGF receptor-expressing cells with cell monolayers expressing pro-TGF-α (Anklesaria *et al,* 1990). Thus, a membrane-anchored growth factor precursor and its receptor can funtion simultaneously as mediators of cell–cell adhesion and as initiators of mitogenic stimulation by cell–cell contact. Cleavage of pro-TGF-α is not aimed at generating the active form of the factor but at switching between

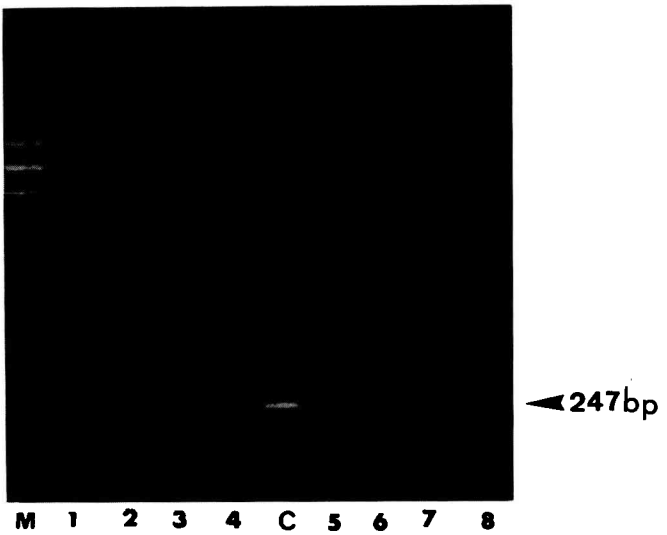

Fig 7. Detection of EGF transcripts within ovine conceptuses (**1–4**) and uterine tissues (**5–8**). Each lane represents the amplifying cDNA aliquot resolved on 2% agarose gel along with 2 μg of 1 kb DNA ladder (**M**), and the positive control (**C**) from human salivary gland which expressed the 247 bp band. The PCR products are as follows: **1**) day 15 conceptus; **2**) day 20 conceptus; **3**) day 25 conceptus; **4**) day 30 conceptus; **5**) day 5 endometrium; **6**) day 15 endometrium; **7**) day 20 endometrium; and **8**) day 25 endometrium.

2 active forms, a membrane-bound and a diffusible form (Massagué, 1990; Massagué and Pandiella, 1993). The regulation of this step would dictate which form of TGF-α is produced by the cell.

Growth factors certainly play a functional role in the development of embryo. *In vitro* production of bovine preimplantation embryo will help to elucidate the role that growth factor genes play during eutherian preimplantation development. When TGF-β and bFGF are added to bovine embryo medium culture, 39% of the embryos can develop through the 8-cell block compared to 0% for the control group (Larson *et al*, 1990). Furthermore, Seidel *et al* (1991) reported that 37% of *in vitro*-cleaved and matured bovine zygotes can develop to the blastocyst stage in a completely defined medium.

We known that the biological activities induced by EGF are also shared by TGF-α in many responses and TGF-α is even a more potent agent than EGF (Partanen, 1990). TGF-α affects the rate of mouse embryo development and blastocyst cavity expansion and is thus postulated to act on the first differentiative step of the preimplantation embryo (Dardik and Schultz, 1991). TGF-α (and also EGF) stimulated the expression of *c*-fos and the incorporation of [^{35}S]methionine into proteins by blastocysts cultured *in vitro* (Werb, 1990).

TGF-α of uterine origin may have an autocrine effect by increasing proliferation of decidual tissue and stimulating decidual hormone (PRL) production in the rat (Han *et al*, 1987). A paracrine role on ovine blastocyst development may be proposed,

but further investigation is required to define whether this potential growth factor plays any role in the supposed embryotrophic effects of endometrial cell coculture on peri-implantation embryo development.

Considering the biological efficiency of growth factors at very low concentrations and the small amounts of peptide probably required to exert paracrine or even autocrine effects, this study emphasizes a positive approach based on RT-PCR method. However, because of the difficulty of quantifying the original tissue levels on mRNA after gene amplification, further improvements are required to quantify PCR, in order to study discrete changes of mRNA levels in small fragments of ovine tissue, during embryogenesis.

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REFERENCES

Alonso S, Minty A, Bourlet Y, Buckingham M (1986) Comparison of three actin-coding sequences in the mouse; evolutionary relationships between the actin genes of warm-blooded vertebrates. *J Mol Evol* 23, 11-22

Anklesaria P, Teixido J, Laiho M, Pierce JH, Greenberger JS, Massagué J (1990) Cell–cell adhesion mediated by binding of membrane-anchored transforming growth factor-α to epidermal growth factor receptors promotes cell proliferation. *Proc Natl Acad Sci USA* 87, 3289-3293

Brachmann R, Lindquist PB, Nagashima M *et al* (1989) Transmembrane TGF-α precursors activate EGF/TGF-α receptors. *Cell* 56, 691-700

Carpenter G, Cohen S (1979) Epidermal growth factor. *Annu Rev Biochem* 48, 193-216

Chomczynski P, Sacchi N (1987) Single step method of RNA-isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162, 156-159

Coffey RJ, Derynck R, Wilcox JN *et al* (1987) Production and auto-induction of transforming growth factor-α in human keratinocytes. *Nature (Lond)* 328, 817-820

Dardik A, Schultz RM (1991) Blastocoel expansion in the preimplantation mouse embryo: stimulatory effect of TGF-α and EGF. *Development* 113, 919-930

DeLarco J, Todaro GJ (1978) Growth factors from murine sarcoma virus-transformed cells. *Proc Natl Acad Sci USA* 75, 4001-4005

Derynck R (1988) Transforming growth factor-α. *Cell* 54, 593-595

Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV (1984) Human transforming growth factor-α: precursor structure and expression in *E coli*. *Cell* 38, 287-297

Derynck R, Jarrett JA, Chen EY, Goeddel DV (1986) The murine transforming growth factor-β precursor. *J Biol Chem* 261, 4377-4379

Downward J, Yarden Y, Mayes E *et al* (1984) Close similarity of epidermal growth factor receptor and v-erb-B. *Nature (Lond)* 307, 521-527

Feinberg AP, Vogelstein B (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 137, 266-267

Gharib-Hamrouche N, Chêne N, Guillomot M, Martal J (1993) Localization and characterization of EGF/TGF-α receptors on peri-implantation trophoblast in sheep. *J Reprod Fertil* 98, 385-392

Goldin GV, Opperman LA (1980) Induction of supernumerary tracheal buds and the stimulation of DNA synthesis in the embryonic chick lung and trachea by epidermal growth factor. *J Embryol Exp Morphol* 60, 235-243

Gray A, Dull TJ, Ullrich A (1983) Nucleotide sequence of epidermal growth factor cDNA predicts a 128 000 molecular weight protein precursor. *Nature (Lond)* 303, 722-725

Gubler U, Hoffman BJ (1983) A simple and very efficient method of generating cDNA libraries. *Gene* 25, 263-269

Han VK, Hunter ES, Pratt RM, Zendegui JG, Lee DC (1987) Expression of rat-transforming growth factor α mRNA during development occurs predominantly in the maternal decidua. *Mol Cell Biol* 7, 2335-2343

Kaplowitz PB, D'Ercole AJ, Underwood LE (1982) Stimulation of embryonic mouse limb bud mesenchymal cell growth by peptide growth factors. *J Cell Physiol* 112, 353-359

Kudlow JE, Leung AWC, Kobrin MS, Paterson AJ, Asa SL (1989) Transforming growth factor-α in the mammalian brain. *J Biol Chem* 264, 3880-3883

Larson RC, Ignotz GG, Currie WB (1990) Defined medium containing TGF-β and bFGF permits development of bovine embryos beyond the "8-cell block". *J Reprod Fertil Abstr* ser No 4

Lee DC, Rose TM, Webb NR, Todaro GJ (1985) Cloning and sequence analysis of a cDNA for rat transforming growth factor-α. *Nature (Lond)* 313, 489-491

Maniatis T, Fristsch EF, Sambrook J (1982) *Molecular Cloning.* Cold Spring Harbor Lab Pub, New York, USA

Massagué J (1983) Epidermal growth factor II. Interaction with epidermal growth receptors in human placental membranes and A431 cells. *J Biol Chem* 258, 13614-13620

Massagué J (1990) Transforming growth factor-α. A model for membrane-anchored growth factor. *J Biol Chem* 265, 21393-21396

Massagué J, Pandiella A (1993) Membrane-anchored factors. *Annu Rev Biochem* 62, 515-541

Mead JE, Fausto N (1989) Transforming growth factor-α may be a physiological regulation of liver regeneration by means of an autocrine mechanism. *Proc Natl Acad Sci USA* 86, 1558-1562

Partanen AM (1990) Epidermal growth factor and transforming growth factor-α in the development of epithelial-mesenchymal organs of the mouse. *Curr Top Dev Biol* 24, 31-55

Pratt RM (1980) Involvement of hormones and growth factors in the development of the secondary palate. *In: Development in Mammals* (MH Johnson, ed) Elsevier North Holand, New York, USA, vol 4, 203-231

Puissant C, Houdebine LM (1990) An improvement of the single-step method of RNA isolation by an acid guanidinium thiocyanate-phenol-chloroform extraction. *Bio-Techniques* 8, 3-4

Rappolee DA, Breener CA, Schultz R, Mark D, Werb Z (1988a) Developmental expression of PDGF, TGF-α, and TGF-β genes in preimplantation mouse embryos. *Science* 241, 1823-1825

Rappolee DA, Mark D, Banda MJ, Werb Z (1988b) Wound macrophages express TGF-α and other growth factors *in vivo*: analysis by mRNA phenotyping. *Science* 241, 708-712

Reed KC, Mann DA (1985) Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res* 13, 7207-7221

Saiki RK, Gelfand DH, Stoffel S *et al* (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487-491

Samsoondar J, Kobrim MS, Kudlow J (1986) α-Transforming growth factor secreted by untransformed bovine anterior pituitary cells in culture. *J Biol Chem* 261, 14408-14413

Seidel GE, Jr, Nauta W, Olson SE (1991) Effects of myoinositol, transferrin and insulin on culture of bovine embryos. *J Anim Sci* 69 (suppl 1) 403, Abstr

Todaro GJ, Fryling C, DeLarco JE (1980) Transforming growth factors produced by certain human tumor cells: polypeptides that interact with epidermal growth factor receptors. *Proc Natl Acad Sci USA* 77, 5258-5262

Tokunaga K, Taniguchi H, Yoda K, Shimuzu M, Sakiyama S (1986) Nucleotide sequence of a full-length cDNA for mouse cytoskeletal β-actin mRNA. *Nucleic Acids Res* 14, 2829

Watson AJ, Hogan A, Hahnel A, Wiemer KE, Schultz GA (1992) Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. *Mol Reprod Dev* 31, 87-95

Watson AJ, Watson PH, Arcellana-Panlilio M *et al* (1994) A growth factor phenotype map for ovine preimplantation development. *Biol Reprod* 50, 725-733

Werb Z (1990) Expression of EGF and TGF-α genes in early mammalian development. *Mol Reprod Dev* 27, 10-15

Wilcox JN, Derynck R (1988) Localization of cells synthesizing transforming growth factor-alpha mRNA in the mouse brain. J Neurosci 8, 1901-1904

Wong ST, Winchell LF, McCune BK *et al* (1989) The TGF-α precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. *Cell* 56, 495-506

Yoneda T, Pratt RM (1981) Mesenchymal cell from the human embryonic palate are highly responsive to epidermic growth factor. *Science* 213, 563-565