BUTTERWORTH HEINEMANN

A RAPID METHOD FOR mRNA DETECTION IN SINGLE-CELL BIOPSIES FROM PREIMPLANTATION-STAGE BOVINE EMBRYOS

M.E. Collins, D.A. Stevens[a], L.J. Jenner[b] and J. Brownlie

Institute for Animal Health, Compton, Newbury, Berkshire, RG16 0NN, UK

Received for publication : *July 18, 1994*
Accepted : *November 30, 1994*

ABSTRACT

Major questions concerning the control of development and gene expression at the cellular level are still unanswered. Nowhere is this more evident than during the earliest stages of development and embryogenesis. This study describes the detection of specific gene transcripts in single cells derived from bovine embryos. Following in vitro fertilization (IVF) and in vitro culture (IVC) of bovine embryos, small groups of cells and even single blastomeres from 32 to 64-cell embryos were micromanipulated into individual tubes for analysis of cytoplasmic RNAs. Reverse transcriptase-PCR was applied to cell lysates for the amplification of β-actin mRNA transcripts. Primers were designed to flank an intron expected to be present within genomic DNA sequences, thus allowing for simple differentiation between DNA- and RNA-derived amplification products. Using a 50-cycle amplification profile, a 260 bp band could be seen as a PCR product derived from a single blastomere following electrophoresis in an ethidium bromide-stained agarose gel. The identity of the band was verified by DNA sequence determination and diagnostic restriction digestion. Lysates derived from single blastomeres in this way have been used for simultaneously phenotyping multiple RNA products. This capability allows the spatial analysis of gene expression and development within embryos from the earliest stages of cellular differentiation.

Key words: RT-PCR, mammalian development, single blastomere, mRNA phenotyping.

INTRODUCTION

The Polymerase Chain Reaction (PCR) has been used in many instances for

---

Acknowledgments

[a] Current address: Royal Brompton National Heart and Lung Institute, Dovehouse Street, London, UK.

[b] Current address: Assisted Conception Unit, St. James's University Hospital, Leeds, UK.

Our thanks are due to Jill Ross for her assistance with the generation and culture of the embryos used and to Helen Prentice who additionally has demonstrated great skill in the micromanipulation of embryos and blastomeres, making this study possible. Stuart Kennedy is gratefully acknowledged for his assistance with the DNA sequencing. This work was funded by the AFRC. David Stevens was supported by a grant jointly funded by the Milk Marketing Board and the Meat and Livestock Commission.

Theriogenology 43:1227–1238, 1995

0093-691X/95/$10.00

genotype analysis. The great power of this technique lies in its sensitivity which allows for the analysis of very small quantities of material. When applied at the single cell level, the PCR of pinched blastomeres from early embryos has allowed for successful differentiation between male and female embryos by amplification of Y-chromosome specific markers (3,8,15) and the determination of particular genotypes for markers such as milk proteins (1). While such analysis of the genome is of great importance in the selection of embryos of a desired genotype, particularly for breeding programs, it tells us nothing about the patterns of gene expression. In many cases it is the temporal or the spatial pattern of gene expression that is of critical relevance to the study of embryonic development, and simple PCR does not answer these questions. Much work has been done using in situ hybridization to investigate mRNA expression in tissue sections and even in whole mount Xenopus embryos (9), but these techniques cannot be easily applied to the study of mammalian embryos with the required degree of specificity and sensitivity. More recently, the application of reverse transcription-PCR (RT-PCR) has been developed as a sensitive technique for analysis of gene expression rather than genotype. In comparison with standard PCR of DNA templates the technique of RT-PCR requires the isolation and enzymic reverse transcription of cellular RNAs prior to amplification. The technical challenge presented by this requirement is not trivial, particularly when applied to the analysis of embryonic mRNA expression. Cellular RNAs are notoriously susceptible to degradation, and due to their single stranded nature they can adopt a high degree of secondary structure, rendering them resistant to reverse transcription except in the presence of toxic denaturants such as methyl mercuric hydroxide. The potential benefits of RT-PCR are significant, particularly for the study of embryonic development. If sufficient sensitivity can be developed RT-PCR will allow for the investigation of patterns of gene expression in cell lineages at the earliest stages of differentiation.

The onset of embryonic transcription has been examined in many species. The effects of the transcriptional blocker α-amanitin on patterns of protein expression in whole embryos has been investigated in the murine by Braude (4) and in the bovine by Barnes and First (2). In addition, Frei et al (6) showed changes in polypeptide synthesis and the beginning of incorporation of radiolabelled uridine into RNA to occur at the 8 to 16-cell stage in the bovine. These data indicate that in bovine embryos the onset of transcription occurs at this time, and it is likely that this increase in endogenous gene activity corresponding with the maternal-zygotic transition is important in the further development of the embryo. However, this does not allow for the identification of specific gene expression, nor does it provide information concerning the key events occurring at this stage in development. The RT-PCR has been used to investigate the expression of a variety of genes in early embryos (7,16,17,20). The possible role of various growth factors and growth factor receptors in the development and differentiation of early embryos has been investigated in both murine and bovine embryos (11,18,20), and significant differences in the patterns of gene expression were noted in bovine and murine embryos at the same stages of development. In these studies pools of in vitro cultured bovine embryos (50 to 150 embryos at each stage examined) were used for mRNA isolation and analysis. While this has provided more precise understanding of the temporal expression of specific

alterations in gene expression such as those that must occur during the differentiation of morulae and blastocysts.

Methods have been described which detect multiple cDNAs from single cell-derived mRNA samples (10,12), but these utilize time-consuming RNA extraction procedures which are prone to the risk of sample loss and/or to multiple step reactions. Given the feasibility of micromanipulation of early embryos and blastomere pinching techniques, we have explored the possibility of using RT-PCR to investigate gene expression in single cells. The aim of this study was to produce a simple, rapid method which would be easily reproducible. To aid the development of this technique to the required degree of sensitivity, we chose β-actin as the target for amplification in the first instance. Thus we present a rapid method for the detection of specific mRNA in individual blastomeres from 16- and 32-cell stage embryos using RT-PCR.

MATERIALS AND METHODS

In-Vitro Production of Embryos

Bovine embryos at the 16 to 32-cell stage were generated for this study by IVF of oocytes recovered from abattoir-derived ovaries. After collection the cumulus-oocyte-complexes were washed twice in TCM-199 with Earles' salts and sodium bicarbonate containing 10% heat-treated adult bovine serum, 50 IU/ml penicillin G and 50 ug/ml streptomycin before being placed into 40 ul microdrops of maturation medium under silicone oil. The maturation medium was a bicarbonate buffered TCM-199 with Earles' salts supplemented with 10% heat-treated estrus cow serum, 12.5 ug/ml estradiol-17β, 2.5 IU/ml Folligon (PMSG; Intervet, Cambridge, UK), 0.4 mM L-glutamine, 0.2 mM pyruvate, 50 IU/ml penicillin G and 50 ug/ml streptomycin. After 24 to 26 h of maturation at 38.5°C in 5% CO_2 in air, the oocytes were inseminated for 24 h in a glucose-free IVF-TALP solution (Earles' balanced salt solution containing 25 mM bicarbonate, 0.2 mM pyruvate, 20 ug/ml heparin, 10 mM caffeine, 10 uM hypotaurine, 10 mM lactate, 50 ug/ml gentamycin and 6 mg/ml BSA). After thawing the spermatozoa were washed twice using sperm-TALP (as IVF-TALP but containing 0.01 M Hepes buffer, 1.25 mM magnesium chloride, 21.5 mM lactate and 1 mM pyruvate) and adjusted to an insemination dose of $2x10^6$/ml in IVF-TALP. After 24 h the inseminated oocytes were co-cultured on monolayers of granulosa cells for a further 5 to 6 d before removal for blastomere pinching. The co-culture drops were prepared 8 d prior to use and contained embryo culture medium (TCM-199 with Earles' salts and sodium bicarbonate supplemented with 10% heat-treated adult bovine serum, 0.4 mM L-glutamine, 0.2 mM pyruvate, 10 mM lactate, 50 IU/ml penicillin G and 50 ug/ml streptomycin).

Embryo Manipulation

Bovine embryos (32 to 64-cell stage of development) were micromanipulated under oil in 40-ul drops of PBS containing 10% heat-inactivated bovine serum, 50 IU/ml penicillin G and 50 ug/ml streptomycin. The embryo was anchored firmly by

microneedle before the introduction of a micropipette. Blastomeres were removed ("pinched") by gentle suction and were transferred directly transferred into 100-ul of medium (PBS containing 10% heat-inactivated bovine serum, 50 IU/ml penicillin G and 50 ug/ml streptomycin) in 0.5-ml microtubes

Extraction and Amplification of mRNA

Pinched blastomeres were washed by serial transfer through 3 x 100 ul serum free PBS at 38.5°C and manipulated into 20 ul RT mix (15 mM $(NH_4)_2SO_4$, 20 mM Tris HCl (pH 8.8), 2 mM $MgCl_2$, 0.05% Tween, 0.05% NP-40) with 0.25 ul RNAguard (Pharmacia Biotechnology, St Albans, UK) and 50 pmol primer A2 in 0.5-ml microtubes. Tubes were then heated in a thermal cycling block (PTC-100, MJ Research Inc., Massachusetts, USA) to 70°C for 5 min to denature the secondary RNA structures and to prevent potential mispriming of subsequent cDNA synthesis. The RT mix was completed with the addition of 5 units of Tth polymerase (Tet-Z, Amersham International, Little Chalfont, UK), 2.5 mM MnCl and 0.8 mM dNTP. The solution was covered with mineral oil and incubated at 60°C for 20 min to allow for reverse transcription of the RNA. The buffer composition was then optimized for DNA amplification by the addition of 80 ul of polymerase buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris HCl (pH 8.8), 1.5 mM $MgCl_2$ and 0.01% Tween-20) containing 50 pmol of primer A1.

The PCR regimen consisted of a denaturation step of 94°C for 2 min, followed by 50 cycles of 92°C for 1 min, 60°C for 2 min (combining both annealing and extension in a single step), and a final cycle of 92°C for 2 min and 60°C for 7 min. The final step held the tubes at a temperature of 6°C until they were removed from the block for analysis.

As a positive control, total RNA extracted from tissue culture cells using the method of Chomcyzinski and Sacchi (5) was amplified in a parallel reaction. A variety of negative controls were included: reactions containing template RNA but no primers, or primers but no template. Particular care was taken to avoid sample contamination since the target (actin mRNA) is ubiquitously present.

RT-PCR Primers

Oligonucleotide primers were prepared using an ABI 392 DNA/RNA Synthesizer (Applied Biosystems, Warrington, UK.). Primer sequences were chosen on the basis of conserved regions in available β-actin gene sequences (mammalian and avian). The sequence of the bovine β-actin gene was not available for comparison. Two primers, A2 (5'-GAGAAGCTGTGCTACGTCGC-3') and A1 (5'-CCAGACAGCACTGTGTTGGC-3') were designed to hybridize on either side of an intron expected to be present in the genomic DNA. It was expected that mRNA amplification products would be in the region of 260 bp, while products of genomic DNA amplification would be 318 bp in size.

General DNA Techniques

Purification and digestion of PCR fragments, gel electrophoresis and other general DNA manipulations were performed according to standard methods (14) or according to protocols supplied by the manufacturer of the reagents. Following amplification, 15 ul of the 100-ul amplification reaction was electrophoresed on a 1.5% or 2% agarose/TBE gel containing 0.5 ug/ml ethidium bromide. To estimate the size of amplified products, 0.5 ug of HaeIII digested PhiX174 DNA molecular weight markers (Promega, Southampton, UK) were also electrophoresed. The fragment sizes of these markers were 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 base pairs, respectively. Before restriction digestion, PCR fragments were column purified using the Magic PCR purification system (Promega) according to the manufacturers protocol. The DNA was eluted in 50-ul water, and an appropriate volume (containing approximately 50ng DNA) was digested with PleI (New England Biolabs, distributed by Cambridge BioScience, UK) at 37°C before gel electrophoresis. To confirm that amplification of the 260 bp band was RNA-dependent, an aliquot of the template solution was incubated with 1 ul of RNAase A (10 mg/ml, Pharmacia Biotech.) prior to amplification. Similarly, to confirm that amplification was DNA-independent, an aliquot of the template material was incubated with 1 ul DNAase I (10 u/ul, Pharmacia Biotech.) at 37°C for 2 h followed by inactivation of the enzyme at 70°C for 10 min prior to amplification.

DNA Sequencing

A 50-ng sample of Magic PCR purified DNA (see previous section) was used for DNA sequence analysis using the primers described above with the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and the model 373A Automated DNA Sequencer according to the manufacturers instructions (Applied Biosystems). Data generated in this way were analyzed using the programs in the GCG DNA sequence analysis package (Genetics Computer Group, Wisconsin, USA, 1991).

RESULTS

Initial attempts at amplification of actin-specific sequences from total cellular RNA extracted from tissue culture cells confirmed the efficacy of both the primers and the amplification protocol. The size of the amplimer and the persistence of specific amplification following pretreatment of the template with DNAase I confirmed the amplimer as being RNA derived. Conversely, similar treatment of the template with RNAase A abolished amplification (Figure 1).

The first attempts at amplification of actin from small numbers of blastomeres were unsuccessful. Similar reactions on comparable dilutions of total RNA derived from known numbers of tissue culture cells produced bands of the expected size, indicating that the interference was due to the blastomeres rather than to a lack of sensitivity of the RT-PCR methodology. Since the blastomere samples were lysed directly in the RT buffer rather than undergoing extensive RNA purification, it was

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.