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Quantification of mRNA in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT–PCR

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Deciphering the complex series of regulatory events that occur during early development depends partly on the ability to accurately quantify stage-specific mRNA species. However, the paucity of biological material coupled with the lack of sensitivity and/or reproducibility of the currently available quantitative methods had been severe limitations on single cell analysis. Rapid cycle DNA amplification is a highly sensitive technique for amplification of specific DNA sequences. With the addition of fluorescence probes, it is possible to monitor the log-linear phase of amplification during which the most useful quantitative data is obtained. Unknown concentrations are extrapolated from standards co-amplified producing a standard curve. Furthermore, micro volume capabilities allow for the analysis of minute samples. Consequently, this approach is ideally suited to the needs of the clinical IVF laboratory. Rapid fluorescence monitored cycling was used to examine expression levels of the housekeeping genes β-*actin* and hypoxanthine guanine phosphorlbosyltransferase in individual murine/human oocytes and/or embryos. Results obtained compared favourably with those attained by others and followed the predicted temporal patterns of expression. Once informative reproductive molecular markers are identified by micro-array analysis, minimally invasive techniques can be developed to biopsy cytoplasm and/or polar bodies for clinical evaluation using rapid fluorescence monitored reverse transcription–polymerase chain reaction methods.

Key words: human oocytes/preimplantation embryos/quantification/rapid cycling/RT–PCR

Introduction

Though many questions remain, some progress has been made toward understanding the molecular mechanisms governing oocyte maturation and preimplantation development. Prior to ovulation, the metabolism of the oocyte is characterized by active gene expression. Subsequent to fertilization, a complex series of gene regulatory events occur that result in fundamental alterations in nuclear transcription (Worrad *et al.*, 1994; Aoki *et al.*, 1997). However, the interplay between the factors mediating development is not yet understood, precluding the elaboration of precise regulatory pathways. Gaining insight into how early developmental processes are controlled and mediated will require specific information regarding molecular events during this period.

Determining the physiological timetable of gene expression during early development depends in part on the ability to accurately measure stage specific mRNA species. Classical techniques of RNA analysis such as $C_o t$ (the product of nucleic acid concentration and time) value assays (Davidson and Hough, 1969), Northern blotting (Thomas, 1980) and dot- or slot-blots (White and Bancroft, 1982) lacked the sensitivity to detect mRNA in single cells and/or present in low copy numbers. Furthermore, these methods provide for crude quantitative analysis at best. Due to its unprecedented sensitivity, the reverse transcription–polymerase chain reaction (RT–PCR) allows the detection of low abundance mRNA in individual cells (Rappolee *et al.*, 1988). RT–PCR used in conjunction with radio-labelled probes has permitted the analysis of gene expression from a small number of embryos (Rambhatla *et al.*, 1995). For a quantitative technique to be deemed reliable, it must be reproducible and precise but, above all, accurate (Ferre, 1992). However, due to differential reaction efficiencies and kinetics, the amount of product obtained from a sample following amplification may not necessarily reflect the initial target concentration. A variety of competitive PCR strategies have been developed to overcome the limitations of endpoint analysis (Becker-André and Hahlbrock, 1989; Wang *et al.*, 1989; Stieger *et al.*, 1991; Sperison *et al.*, 1992). However, these methods are tedious requiring numerous dilution series and the construction of a different competitor for every target to be quantified.

Realtime fluorescence monitored PCR offers both a fast and sensitive quantification solution. Higuchi *et al.* (1993) pioneered realtime PCR analysis by introducing fluorescent dyes in the reaction to monitor product accumulation. Double-stranded DNA (dsDNA) specific dyes such as Sybr Green I (Molecular Probes, Eugene, OR, USA) are simple to use and permit generic product identification. By monitoring fluorescence as the reaction progresses, it is possible to identify the threshold cycle or the cycle during which fluorescence rises above background for each sample. The most reliable data for quantification is obtained at the threshold cycle during the log-

linear phase of the reaction. Unknown concentrations are extrapolated from the threshold cycles of titered known quantities amplified in the same reaction producing a standard curve (Wittwer *et al.*, 1997c). With additional enhancements to the PCR method such as rapid cycle DNA amplification, specificity and yield was improved (Wittwer and Garling, 1991) minimizing the need for nested amplifications. Together with micro volume capillaries, this method allows for the study of extremely minute samples (Wittwer *et al.*, 1997b). The suitability of this technique for the examination of gene expression in individual oocytes and embryos has been confirmed (Steuerwald *et al*., 1999).

The aim of this investigation is to demonstrate the utility of fluorescence monitored RT–PCR for quantitative analysis of gene expression during early development. The rationale for these experiments is to develop rapid real-time methods to quantify copy number in mouse/human oocytes and subcellular components thereof for clinical evaluation. We applied this technique to the examination of expression levels of the housekeeping genes β-*actin* and hypoxathine guanine phosphorlbosyl transferase (*HPRT*) in individual murine oocytes and embryos. These messages were selected because they are abundantly and moderately expressed, respectively, (Bishop *et al*., 1974; Getz *et al*., 1975). Furthermore, they undergo established fluctuations in expression levels throughout mouse development (Paynton, 1988; Bachvarova, 1989). Consequently, their analyses would permit the assessment of the degree of sensitivity of this technique. Resulting mRNA amounts are contrasted with those obtained by previous investigators using classical methods that were extrapolated from pooled material. Furthermore, quantitative analysis was conducted using human oocytes in order to determine if similar temporal patterns and corresponding levels of expression are discernable.

Materials and methods

Oocytes and embryos

Spare human oocytes were obtained from patients undergoing assisted reproduction at The Institute for Reproductive Medicine and Science of Saint Barnabas following written consent and Institutional Review Board approval. Oocytes used in this study (n = 22) consisted of discarded immature oocytes (metaphase I; MI) or mature oocytes (metaphase II; MII) that failed to fertilize following insemination.

Mouse oocytes and blastocysts were obtained from CB6F1 female mice in which ovulation had been stimulated using 10 IU pregnant mare's serum (PMS; Sigma, St Louis, MO, USA) followed 49 h later with 10 IU human chorionic gonadotrophin (HCG; Sigma). To obtain blastocysts, the animals were immediately placed to mate with males. The females were killed by cervical dislocation upon detection of the copulation plug. Embryos were flushed from the excised oviducts and cultured for ~96 h in KSOM culture medium (Cell and Molecular Technologies Inc, Lavallette, NJ, USA).

RNA isolation

Total RNA was isolated from individual oocytes and embryos using a Micro RNA Isolation Kit (Stragene, La Jolla, CA, USA) according to the manufacturer's instructions except for the addition of 10 µg glycogen (Boehringer Mannheim, Indianapolis, IN, USA) as carrier prior to precipitation with isopropanol. A fixed amount of exogenous RNA transcribed from the synthetic plasmid pAW109 (Perkin Elmer, Foster City, CA, USA) was added to each sample. The pAW109 RNA includes sequences complementary to those present in the plasmid insert. The insert contains a synthetic linear array of primer sequences for multiple targets constructed such that upstream primer sites are followed by sequences complementary to their downstream primer sites in the same order. The pAW109 RNA template was added to serve as a control for RNA recovery and reverse transcription.

Table I. Polymerase chain reaction (PCR) primer sequences

PCR primers	
Human β-actin	5′-GGCCACGGCTGCTTC-3′
	5′-GTTGGCGTACAGGTCTTTGC-3′
Mouse β-actin	5′-TGCGTGACATCAAAGAGAAG-3′
	5′-CGGATGTCAACGTCACACTT-3′
Human *HPRT*	5′-GACTTTGCTTTCCTTGGTCA-3′
	5′-GGCTTTGTATTTTGCTTTTCC-3′
Mouse *HPRT*	5′-AAACTTTGCTTTCCCTGGTTA-3′
	5′-AGGCTTTGTATTTGGCTTTTC-3′

Reverse transcription

First-strand complementary DNA synthesis was performed by priming with oligo-dT_{16}. The lyophilized samples were redissolved in an 8.5 µl solution consisting of 1 µl 50 µmol/l oligo-dT_{16}, 0.2 µl 0.1 mol/l dithiothreitol (DTT), 0.05 µl RNase inhibitor (20 IU/µl) (RNasin®; Promega, Madison, WI, USA) and 7.25 µl sterile nuclease-free water. The primers were annealed by incubating the samples to 70°C for 6 min and immediately quenched on icy water for 1 min. Reverse transcription was performed by the addition of 11.5 µl containing 4 µl 25 mmol/l $MgCl_2$, 2 µl 10× PCR buffer II (Perkin Elmer, Foster City, CA, USA), 4 µl dNTP 2 mmol/l, 1 µl RNase inhibitor (20 IU/µl), and 0.5 µl Maloney murine leukaemia virus (MMLV) reverse transcriptase (Gibco BRL, Grand Island, NY, USA) and incubated at 37°C for 60 min. The reaction was stopped by heating to 95°C for 5 min. One µl of final product was used directly for PCR. Concurrently, commercially available liver total RNA, 1 µg (Clonetech, Palo Alto, CA, USA) was processed as a positive control.

Primer and probe design

Complementary DNA PCR primers for human and mouse were designed using Oligo primer analysis software (National Biosciences Inc, Plymouth, MN, USA) from DNA and RNA sequences obtained from GenBank (Benson *et al*., 1998) for β-*actin* (Ponte *et al.*, 1984; Tokunaga *et al.*, 1986) and *HPRT* (Konecki *et al.*, 1982; Jolly *et al.*, 1983). Primer (Gibco BRL) sequences are presented in Table I.

PCR

PCR was performed using a Light-cycler™ (Wittwer *et al.*, 1997b), a combination microvolume fluorimeter and rapid temperature cycler (Idaho Technology Inc, Idaho Falls, ID, USA). The reaction mixture consisted of cDNA, 0.5 µmol/l each primer, 200 µmol/l each dNTP, 2–4 mmol/l $MgCl_2$, 50 mmol/l Tris–HCl, 500 ng/µl BSA, 0.05 IU/µl *Taq* DNA polymerase, and 11 ng/µl TaqStart antibody (Clontech, Palo Alto, CA, USA). In addition, the double-stranded DNA dye, SYBR Green I, 1:3000 of 10 000× stock solution (Molecular Probes, Eugene, OR, USA) was included in each reaction. A 7 µl volume was loaded into the glass micro-capillary reaction vessels. The cDNA was denatured by heating to 96°C for 1 min. Template was amplified by 35–50 cycles of denaturation for 0 s at 95°C, annealing of primers at 50°C (for *HPRT*) or 60°C (for β-*actin*) for 0 s and extension at

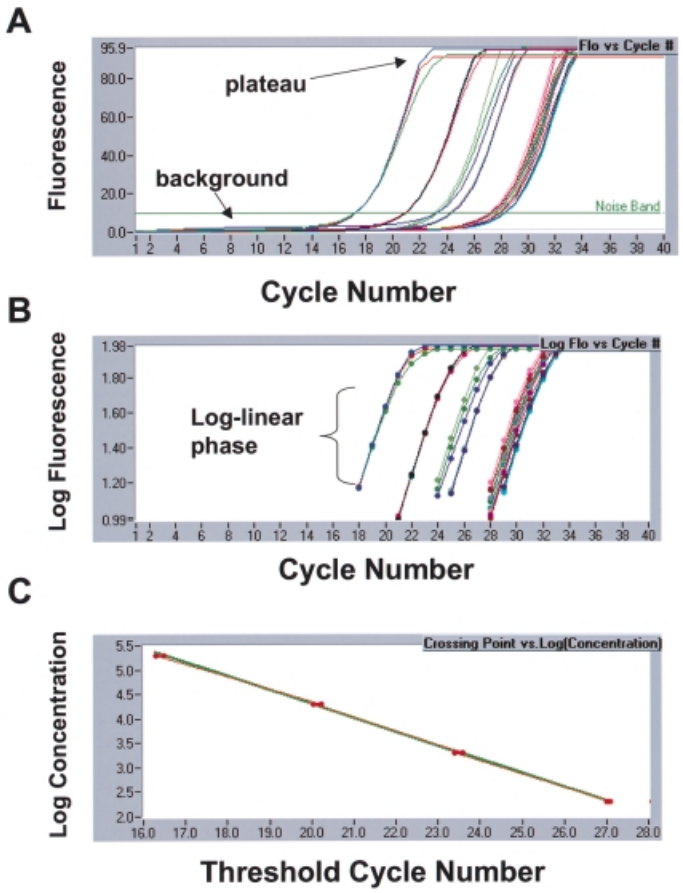

Figure 1. Obtaining sample copy numbers. (**A**) The most useful data for quantification is obtained between the background and the plateau at the crossing point of the logarithmic phase with the noise-band parallel to the *x* axis or at the threshold cycle. (**B**) When these cycles are plotted as the log of fluorescence versus cycle number, they define a straight line. (**C**) Plotting the threshold cycle numbers of titrated known concentrations versus the log of the starting copy number generates a standard curve. The Lightcycler™ quantification software generates a best-fit line and computes the unknown sample concentrations by interpolating their noise-band intercepts against the standard curve (Wittwer *et al.*, 1997c).

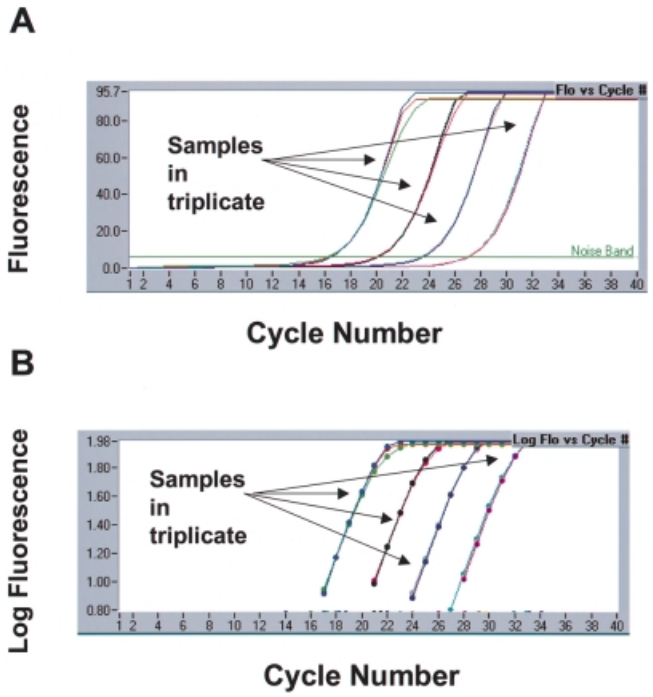

Figure 2. Sample-to-sample reproducibility. Standards which consisted of serial dilutions of gel purified polymerase chain reaction products were amplified in triplicate to produce a standard curve. The efficiency of amplification can be gauged by comparing the threshold cycles and slopes of the amplification curves. Samples that amplify with similar efficiency and have the same starting copy number have the same slope during the log-linear phase of amplification and will have the same threshold cycle (Wittwer *et al.*, 1997c). (**A**) The amplification curves are so similar that they are superimposed for each of the four individual standards points in this reaction. (**B**) The log-linear phase and threshold cycles are also essentially identical.

72°C for 10–15 s. Fluorescence data was acquired during an additional step at ~3°C below the product melting temperature (T_m) for 2 s. Melting curves were plotted to determine PCR product identity. Since the melting curve of a product is sequence specific, it can be used to distinguish them (Ririe *et al*., 1997). A melting curve is produced by monitoring fluorescence continuously while slowly heating the samples in order to observe the loss of fluorescence at the denaturation temperature. The melting protocol consisted of heating the samples to 96°C followed by cooling to 50°C and slowly heating at 0.2°C/s to 97°C while monitoring fluorescence. The curve is then redrawn as the negative derivative of fluorescence with respect to temperature to generate a melting peak. Product identity was confirmed by ethidium bromide-stained 2% agarose gel electrophoresis and verified by sequencing with the murine and human β-*actin* and *HPRT* coding sequences.

Quantification

A standard curve was generated by amplifying serial dilutions of a known quantity of amplicons. Amplicons consisted of purified PCR products which were gel purified using QIAquick gel extraction kit as per the manufacturer's protocol (Qiagen Inc, Valencia, CA, USA). The copy number of the standards was then determined by measuring absorbance at 260 nm (Pharmacia, Piscataway, NJ, USA). The standards in triplicate and cDNA samples were then co-amplified in the same reaction prepared from a master mix. Fluorescence was acquired at each cycle in order to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence rises above background for each sample. The Lightcycler™ quantification software generates a best-fit line and determines unknown concentrations by interpolating the noise-band intercept of an unknown sample against the standard curve of known concentrations (Wittwer *et al.*, 1997c) (Figure 1).

Results

Prior to inclusion in the study, successful amplification from pAW109-derived cDNA was confirmed. Amplification of cDNA synthesized from the exogenous RNA template added to every sample served as a positive control to monitor RT–PCR experiments. Furthermore, the extent of material loss during RNA isolation was assessed by comparison to a 'reverse transcription only' control containing the identical copy number of the exogenous template added to each sample. The standard curves and best-fit lines were generated from a minimum of four points run in triplicate spanning the anticipated unknown values. Individual experiments were repeated a minimum of

Table II. Concentration of β-actin in single mouse oocytes and embryos

Stage	Average copy number	SD	Dilution factor	Final concentration
GV	1715.92	463.97	1:20	3.43E+04
MI	1053.48	297.30	1:20	2.11E+04
MII	1172.76	301.52	1:20	2.35E+04
Blastocyst	34826.25	7948.65	1:20	6.97E+05

GV = germinal vesicle; MI = metaphase I; MII = metaphase II.

Table III. *HPRT* copy number in single mouse oocytes

Stage	Average copy number	SD	Dilution factor	Final concentration
GV	294.11	62.41	1:20	5.88E+03
MI	197.78	43.79	1:20	3.96E+03
MII	241.46	31.59	1:20	4.83E+03

GV = germinal vesicle; MI = metaphase I; MII = metaphase II.

three times to evaluate the degree of variation. The threshold values of the triplicate standard samples were very consistent both within the same reaction and between identical experiments (Figure 2).

Copy numbers obtained following examination of the murine β-*actin* gene are presented in Table II. Quantitative analysis was performed on oocytes at the germinal vesicle (GV), MI and MII stages of maturation as well as on blastocysts. The numbers reflect the average value calculated following three separate experiments with the same samples. The numbers computed for identical samples in separate reactions were very reproducible. Moreover, the values estimated for different samples at the same stage of development were very similar. Therefore, the data were pooled before a final average was computed. The temporal pattern of expression of actin during oocyte maturation has been examined extensively (Paynton *et al*., 1988, 1994; Bachvarova *et al*., 1989; Taylor and Piko, 1990; Temeles *et al*., 1994; Rambhatla *et al*., 1995). The number of actin transcripts we detected in GV oocytes is analogous to the formerly reported copy numbers of 3.6×10^4 (Taylor and Piko, 1990) and 4.0×10^4 (Bachvarova *et al*., 1989). Previous investigators have found that oocytes undergo a decline in actin expression during maturation. We observed a 32% decrease in actin content between GV and MII oocytes which is similar to the 30% reported by Paynton *et al*. (1988). Our estimated actin transcript number for the blastocyst is 21-times higher than our value for the GV oocyte. This result compares favourably with the 27-fold and 34-fold increase observed by Bachvarova *et al*. (1989) and Rambhatla *et al*. (1995) respectively.

Results from the quantitative analysis of the murine *HPRT* gene are presented in Table III. Murine oocytes were examined for *HPRT* expression at the GV, MI and MII stages of maturation. Again, data were pooled because the degree of variation both within and between samples at the same stage of development was comparable. Our value for *HPRT* content in GV oocytes was similar (5.9×10^3 versus 7.4×10^3) to the value obtained by Rambhatla *et al*. (1995). We observed a minor decrease in *HPRT* concentration between GV and MII oocytes (17.9%) as did these investigators (13.5%).

Table IV. β-actin concentration in individual human oocytes

Stage	Average copy number	SD	Dilution factor	Final concentration
GV 1	31193.33	3408.43	1:20	6.24E+05
GV 2	17330.00	1305.41	1:20	3.47E+05
GV 3	1161.80	221.97	1:20	2.32E+04
GV 4	892.30	200.01	1:20	1.78E+04
GV 5	4154.00	1481.49	1:20	8.31E+04
GV 6	1264.33	126.14	1:20	2.53E+04
MI 1	778.90	185.97	1:20	1.56E+04
MI 2	2599.66	373.68	1:20	5.20E+04
MI 3	571.90	176.76	1:20	1.14E+04
MI 5	1042.73	60.25	1:20	2.09E+04
MI 6	7347.00	1540.02	1:20	1.47E+05
MII 1	320.53	5.26	1:20	6.41E+04
MII 2	3852.66	217.29	1:20	7.71E+04
MII 3	1059.23	210.82	1:20	2.12E+04
MII 4	1537.66	105.29	1:20	3.08E+04
MII 5	491.06	65.58	1:20	9.82E+03
MII 6	493.46	121.63	1:20	9.87E+03

GV = germinal vesicle; MI = metaphase I; MII = metaphase II.

Table V. Copy number of *HPRT* in individual human oocytes

Stage	Average copy number	SD	Dilution factor	Final concentration
GV 1	759.70	47.81	1:20	1.52E+04
GV 2	586.13	82.87	1:20	1.17E+04
GV 3	774.80	60.34	1:20	1.55E+04
GV 4	788.00	188.74	1:20	1.58E+04
GV 5	614.06	79.52	1:20	1.23E+04
GV 6	694.26	50.59	1:20	1.39E+04
MI 1	235.96	27.93	1:20	4.72E+03
MI 2	288.45	68.37	1:20	5.77E+03
MI 3	292.33	17.17	1:20	5.85E+03
MI 5	500.03	64.27	1:20	1.00E+04
MI 6	838.95	22.13	1:20	1.68E+04
MII 1	610.95	60.03	1:20	1.22E+04
MII 2	131.00	13.65	1:20	2.62E+03
MII 3	526.83	122.29	1:20	1.05E+04
MII 4	836.53	59.52	1:20	1.67E+04
MII 5	239.83	42.86	1:20	4.80E+03
MII 6	308.63	23.65	1:20	6.17E+03

GV = germinal vesicle; MI = metaphase I; MII = metaphase II.

Copy numbers were estimated for β-*actin* (Table IV) and *HPRT* (Table V) in human oocytes at the GV, MI and MII stages of maturation. Unlike the mouse material, the degree of variation was substantial between individual human samples. However, the numbers obtained for the identical specimens remained consistent. Therefore, only the numbers for identical samples were pooled prior to calculating an average. Nonetheless, the temporal patterns of expression observed in the human samples generally corresponded to that predicted by the mouse data as well as with the findings of others using human material (Heinkinheimo, *et al*., 1995; Daniels *et al*., 1997; Liu *et al*., 1997).

Discussion

Successful quantification of transcripts in individual embryos and oocytes by real-time rapid cycle fluorescence monitored

RT–PCR, as shown by our results, demonstrates the suitability of this approach for the study of early developmental processes. The close correlation of our data with results obtained by other methods lends credence to the efficacy of this technique. The credibility of this method is further substantiated by the reproducible, consistent quantitative data that we obtained for individual samples and standard points.

A serious limitation of early quantitative PCR strategies has been their reliance on endpoint analysis. Since variable reaction efficiencies can alter the duration of the log-linear phase of amplification, considerable differences in overall PCR product synthesis may be observed which may not necessarily correlate with input template concentrations (Piatak *et al.*, 1993). Competitive PCR strategies were developed to overcome this shortcoming. However, these techniques are quite demanding since numerous dilution series are required to synchronize the linear range of amplification between the unknowns and the competitors. Furthermore, a unique competitor must be synthesized for each target to be studied. Quantitative strategies based on continuous monitored PCR eliminate the need for such approaches by permitting real-time examination of the exponential phase of amplification. Consequently, the development of quantitative assays is considerably simplified.

Previous investigators have identified essential factors that influence the outcome of quantitative analysis using the PCR method (Ferre, 1992; Morrison *et al.*, 1998). Primary among these is the need to achieve an optimized reaction. This is especially true in a system that relies on continuous fluorescence observations using Sybr Green I. Since Sybr Green I is a dsDNA dye, it binds generically. Therefore, nonspecific products as well as primer dimer will contribute to the overall fluorescence. Obviously, any quantitative data obtained under such circumstances would not be accurate. This problem can be circumvented by acquiring fluorescence at ~3°C below the specific amplicon's T_m (Morrison *et al*., 1998). Furthermore, spurious amplification from non-specific targets can be minimized by employing hot start strategies (Ferre, 1992; Morrison *et al*., 1998). Alternately, sequence-specific fluorescent hybridization probes could be used to monitor product accumulation (Kramer and Tyagi, 1996; Wittwer *et al.*, 1997a). However, each target to be analysed would require the design and synthesis of a unique probe that can be challenging and expensive.

In addition to these considerations, we have encountered additional concerns that must be taken into account to ensure accurate quantification. The selection of primers for both the RT and PCR reaction can alter quantitative outcome by our method. In particular, the choice of oligomer to prime the RT reaction can modulate the pool of template available to the PCR reaction due to the differential processivity and efficiency of the reverse transcriptase. For example, when using oligo-dT priming for first strand synthesis, the use of primers that bind far upstream of the poly-A tail would not be advisable. Though product may still be detected in such circumstances, the numbers obtained may not be an accurate reflection of mRNA concentration. Alternately, random priming may not yield as high a concentration of template with PCR primers that bind very close to the 3′ end. Likewise, amplicon length can affect quantitative results if the template pool has been biased during first-strand cDNA synthesis. Thus, we selected primers that produced amplicons ~300 bp in length situated close to the poly-A site while dT priming our RT reaction. The selection of amplicons of this length is also desirable if the genomic structure is not known, as exons greater than this size are fairly rare in vertebrates (Hawkins, 1988). Thus, the use of primers spanning introns is assured allowing for detection of genomic DNA contamination following the PCR reaction.

The orderly progression through the stages of oocyte maturation and embryonic development belies the intricate interactions orchestrated by the repertoire of genes being expressed and/or silenced. Little is known of these transcripts, let alone of their functions. The ability to accurately measure mRNA content in individual cells may allow us to begin to dissect the role these messages play during oogenesis and embryogenesis. We plan to investigate the cause of the disparity in expression levels we observed in the human samples. This disparity between individual samples may be attributed to oocyte quality, as the specimens examined were discarded material that is potentially compromised. Patient aetiology may also contribute to the discrepancies. Conceivably, quantitative expression of key molecular markers may influence developmental potential. Our goal is to use rapid real-time RT–PCR fluorescent methods to quantify copy number in mouse/human oocytes and sub-cellular components thereof for clinical evaluation. Micro-array analysis will be necessary to screen for clinically useful reproductive markers. Once identified, a diagnostic assay could be developed to predict enhanced developmental prognosis. Concomitantly, minimally invasive techniques can be developed to biopsy cytoplasm and/or polar bodies for analysis.

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