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Quantitative RT-PCR Amplification of RNA in Single Mouse Oocytes and Preimplantation Embryos

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ABSTRACT

*We describe a simple whole-cell method for quantitative reverse transcription (RT) PCR amplification of RNA that consistently allows the analysis of trace amounts of RNA, such as those carried by a fraction of a single mouse oocyte or preimplantation embryo, without organic extraction. The method is based on a preliminary genomic DNA digestion by DNase I in the presence of Mn^{++} and a subsequent RT step with rT*h *Reverse Transcriptase at 70°C with the same buffer components, which also has the effect to irreversibly denature DNase I activity. Because of the completeness of genomic DNA digestion and RNA recovery, this procedure makes it possible to quantitatively amplify any target RNA, including those coded by intronless genes or genes whose intron-exon boundaries are unknown. By taking mRNAs of b-actin, heat-shock protein HSP70.1 and ribosomal protein S16 as experimental models, we demonstrate the effectiveness of genomic DNA digestion by DNase I-Mn^{++} and of DNase I heat-denaturation and the quantitative properties of our method. We also show that this procedure is useful for transcriptional analyses during development that are hindered by paucity of biological material.*

INTRODUCTION

Because of its sensitivity, reverse transcription-polymerase chain reaction (RT-PCR) RNA amplification has rapidly become the method of choice for detecting and measuring rare messages. This procedure can also be scaled down to the level of a few or single cells (17) and performed on crude whole-cell lysates (12). In the latter case, RNA recovery is complete, but the presence of genomic DNA may give false-positive signals. This problem can be overcome by different strategies. First, oligodeoxyribonucleotide primers can be designed appropriately to span an intron and thus produce different-sized DNA and RNA amplification bands. Unfortunately, this strategy requires the genes whose transcript is examined to have been cloned and sequenced. It thus cannot be applied when intron-exon boundaries are still unknown or when the RNA to be amplified is coded by an intronless gene. This is the case, among others, of many heat-shock genes. Second, amplification of RNA, but not DNA, can be achieved by the use of a poly(dT) antisense primer that anchors to the message's poly(A) tail (6). In most cases, however, such strategy limits message amplification to the few hundreds of nucleotides that are close to the poly(A) tail, leaving the message 5′ region virtually inaccessible to investigation. Third, RNA preparation can be preliminarily treated with DNase. Problems in this case will include the control of DNase digestion completeness and the removal/inactivation of the enzyme before the RT step. This is usually performed by extracting the DNase I-treated lysate with organic solvents or by using bead-immobilized DNase I (16,17). In both cases, however, RNA recovery may not be complete because of the necessary transfer of the supernatant/column eluate to another tube.

We have solved this problem by treating the crude, whole-cell lysate with pancreatic DNase I in the presence of Mn^{++} (2) and then irreversibly inactivating this enzyme with the 70°C incubation needed for RT by rT*th* Reverse Transcriptase. In this paper, such strategy has been optimized for quantitative RNA analysis on mouse oocytes and preimplantation embryos on a single-cell basis. This was particularly worth pursuing because, in spite of the relevance that these cells have for both basic and clinical research in humans and domestic mammals, to our knowledge, no RT-PCR method allowing message analysis to be performed on individual mammalian oocytes or early embryos has been reported so far. In fact, previous RT-PCR studies on these cells (9,13–15) used pools of 40–200 oocytes or embryos and were mostly based on RNA extraction-precipitation procedure. We show here that our procedure is consistent, quantitative and sensitive enough to determine the

amounts of β-actin, heat-shock protein HSP70.1 and ribosomal protein S16 mRNAs contained in a fraction of a single mouse oocyte or preimplantation embryo. This now makes it possible to analyze transcripts of experimentally manipulated mammalian oocytes, preimplantation embryos or single embryo blastomeres.

MATERIALS AND METHODS

Cells

Growing and fully grown dictyate mouse oocytes were obtained by puncturing ovarian follicles of prepuberal and hormone-primed adult CD1 mice (Charles River Italia, Calco, Italy), respectively, and were then mechanically freed from surrounding granulosa cells. Metaphase II oocytes and two-cell embryos were collected from the tubes of adult mice and freed from cumulus cells by hyaluronidase, as needed. Individual, or groups of 5–10, oocytes or embryos were transferred to a 0.5-mL Eppendorf® tube (Brinkmann Instruments, Westbury, NY, USA) tube containing 2 µL of embryo-quality H_2O (Sigma-Aldrich S.r.l., Milano, Italy) supplemented with 1 U/µL RNasin® Ribonuclease Inhibitor (Promega, Madison, WI, USA). The tube was immediately frozen on dry ice and stored at -80°C until use.

DNase I-Mn^{++} Incubation

Immediately before the assay, cells were lysed by rapidly thawing and freezing the tube twice. The lysate was then diluted by addition of the following components (final concentrations): 180 mM KCl, 20 mM Tris-HCl, pH 8.3, 2 mM Mn^{++}, 40 U/mL RNase-free bovine pancreatic DNase I (Boehringer Mannheim Italia, Milano, Italy) and 2 U/µL RNasin (DNase-Mn^{++} digestion buffer). This buffer was routinely made using concentrated buffers of a GeneAmp® Thermostable rT*th* Reverse Transcriptase RNA PCR Kit (Perkin-

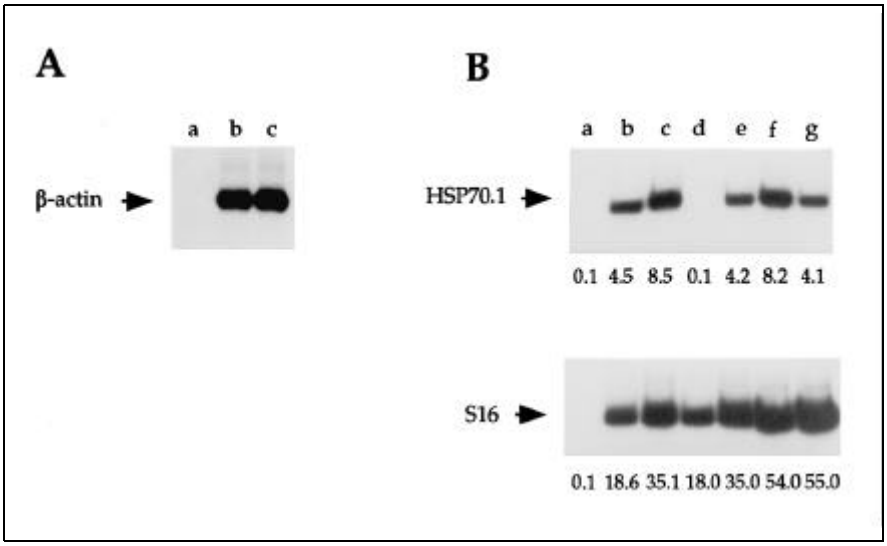

Figure 1. Effectiveness of DNase I digestion. (A) 20 ng of murine β-actin cDNA were subjected to the entire RT-PCR procedure, with the following modifications. From left to right: (a) the sample was incubated with DNase I at 37°C for 5 min; (b) the sample was incubated as described above, in the absence of DNase I; (c) the reaction was assembled with DNase I, but the incubation at 37°C for 5 min was omitted. (B) Two- and four-cell embryos were pooled and subjected to RT-PCR amplification of their HSP70.1 and S16 mRNAs. Pools were as follows: (a) no embryos were present; (b) 5 two-cell embryos; (c) 10 two-cell embryos; (d) 5 four-cell embryos; (e) 5 two-cell embryos and 5 four-cell embryos; (f) 10 two-cell embryos and 5 four-cell embryos; and (g) 5 two-cell embryos and 10 four-cell embryos. Oligonucleotide primer pairs were as follows: β-actin (amplification fragment, 359 bp), 5′-GGTTCCGATGCCCTGAGGCTC-3′ (683–703 nucleotides (nt), sense), 5′-ACTTGCGGTGCACGATGGAGG-3′ (1042–1022 nt, antisense); S16 (amplification fragment, 103 bp), 5′-AGGAGCGATTTGCTGGTGTGGA-3′ (1795–1816 nt, sense), 5′-GCTACCAGGGCCTTTGAGATGGA-3′ (1986–1964 nt, antisense); HSP70.1 (amplification fragment, 239 bp), 5′-GAAGGTGCTGGACAAGTGC-3′ (2509–2527 nt, sense), 5′-GCCAGCAGAGGCCTCTAATC-3′ (2748–2729 nt, antisense). Numbers indicate ^{32}P incorporation into amplification bands (counts per minute [cpm] × 10^{-3}).

Elmer Italia, Monza, Italy). The final volume of DNase-Mn^{++} digestion buffer of each tube depended on the number of assays planned to be performed on that lysate with a 5-µL aliquot for each assay. A typical DNase incubation volume (allowing the subsequent amplification of two different messages and of their control tubes without the RT step) was 20 µL. Genomic DNA digestion was routinely performed by incubating the tubes at 37°C for 5 min. Such time was selected for the sake of confidence, since it largely exceeded the 2–3-min incubation duration that preliminary experiments had shown to be sufficient for the full digestion of an embryo's DNA (not shown).

RT-PCR

For the RT step, the DNase-digested lysate was divided into 5-µL aliquots, as mentioned above, and each aliquot was further diluted to a final volume of 10 µL by addition of a solution containing a mixture of the four dNTPs (giving a 0.2 mM final concentration of each nucleotide), 50 pmol antisense primer, 4 U rT*th* Reverse Transcriptase (from either Perkin-Elmer Italia or Pharmacia Biotech Italia, Cologno Monzese, Italy) and the appropriate amounts of H_2O supplemented with 1 U/µL RNasin. Samples were then incubated at 70°C for 15 min and eventually returned to the ice bucket. Control tubes were constantly maintained at 0°–4°C.

For PCR, each tube was further processed according to the manufacturer's instructions for the GeneAmp Thermostable rT*th* Reverse Transcriptase RNA PCR Kit, with minor modifications. In detail, each tube received a 40 µL mixture containing 4 µL of 10× Chelating Buffer (50% [vol/vol] glycerol, 100 mM Tris-HCl, pH 8.3, 1 M KCl, 0.5% [wt/vol] Tween® 20 and 7.5 mM EGTA), 3 µL of 25 mM $MgCl_2$ solution (giving a final Mg^{++} concentration of 1.5 mM), 50 pmol sense primer and 5 µCi $^{32}P[\alpha$-dCTP] (Du Pont Italiana, Cologno Monzese, Italy) as tracer. PCR was routinely performed in a MiniCycler™ (MJ Research, Watertown, MA, USA), with a first denaturation step at 94°C for 2 min, followed by 34 cycles with a denaturation step at 94°C for 1 min and an annealing-elongation step at 62°C for 1 min. In the case of control tubes lacking the RT step, DNase I was fully denatured by the first step at 94°C for 2 min (not shown). Control experiments, in which identical RNA amounts were subjected to increasing numbers of PCR amplification cycles, indicated that under our conditions, cDNA amplification was exponential for at least 40 cycles (not

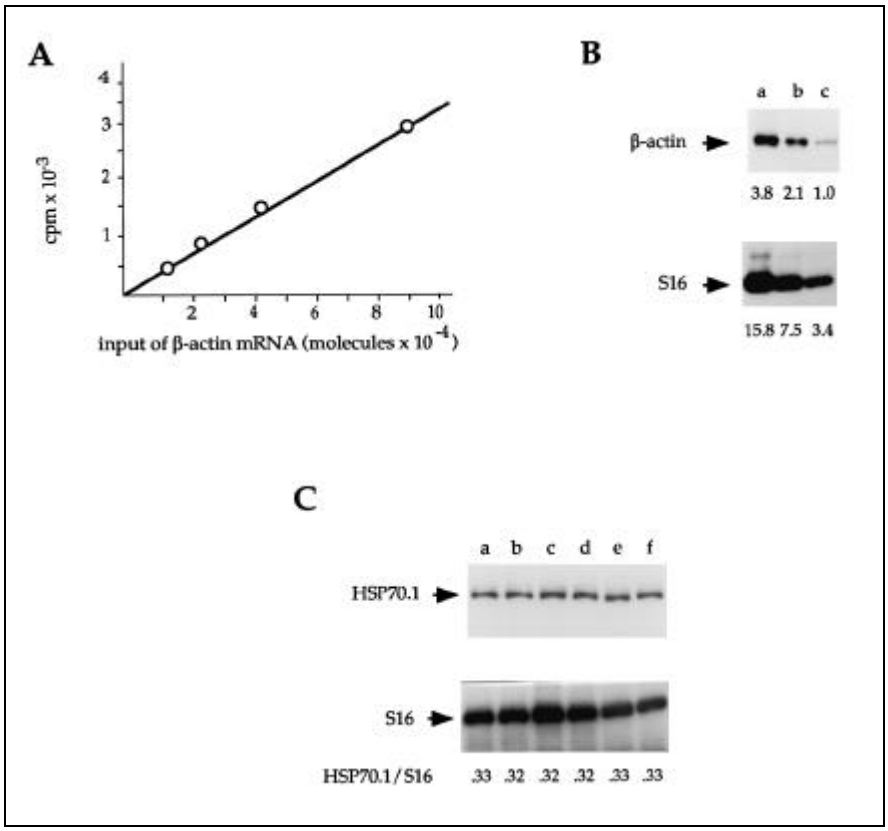

Figure 2. Quantitative properties of RT-PCR method. (A) Relationship between input of murine β-actin mRNA and radioactivity incorporation into RT-PCR-amplified fragment obtained in a typical experiment. (B) Relationship between input of growing oocyte lysate and RT-PCR amplification of β-actin and S16 mRNAs. RT-PCR was performed with decreasing amounts of the same lysate, corresponding for each message to: (a) 2.5 oocytes; (b) 1.25 oocytes; and (c) 0.62 oocytes. (C) Single two-cell mouse embryos were subjected to RT-PCR amplification of their HSP70.1 and S16 mRNAs. Oligonucleotide primer pairs were as reported in the Figure 1 legend. Numbers indicate ^{32}P incorporation (cpm × 10^{-3}) into amplification bands (B) and HSP70/S16 incorporation ratio (C).

shown). Amplification products were fractionated by 5% polyacrylamide gel electrophoresis, and their ^{32}P incorporation was measured with a Model A2024 InstantImager™ (Camberra Packard, Milano, Italy).

RESULTS AND DISCUSSION

We have determined the properties of our RT-PCR procedure by performing several quantitative analyses of β-actin, HSP70.1 heat-shock protein and S16 ribosomal protein mRNAs in mouse growing oocytes and preimplantation embryos. These messages were selected for this study because they undergo well-characterized variations in amount during mouse oogenesis and early embryo development and thus represent definite standards for quantitative analysis of small RNA amounts. β-actin mRNA is abundant in growing oocytes; it then undergoes a sudden, 50% decrease in fully grown oocytes and does not vary further during oocyte maturation (1). HSP70.1 mRNA is transiently synthesized in two-cell mouse embryos and thereafter is completely absent at the four-cell stage (4,5,7). S16 mRNA progressively increases during early oocyte growth, following the developmental pattern of ribosomal protein synthesis in mouse oogenesis (10), and does not significantly vary in late-growing, fully grown and maturing oocytes and in the early cleaving embryos (M.T. Fiorenza, unpublished). Because of the developmental constancy of its content in late oocytes and early embryos, S16 mRNA was used as the internal standard in this study, as previously performed in somatic tissues (8).

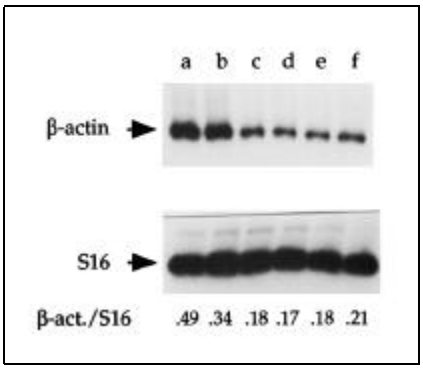

Figure 3. Developmental pattern of β-actin mRNA accumulation into mouse oocytes. Single oocytes at different stages of oogenesis were subjected to RT-PCR of their β-actin and S16 mRNAs. Lanes a–b, growing oocytes; lanes c–d, fully grown oocytes; and lanes e–f, metaphase II oocytes. Numbers indicate β-actin/S16 incorporation ratios of each oocyte. Oligonucleotide primer pairs were as reported in the Figure 1 legend.

Effectiveness of DNA digestion by DNase I under present Mn^{++} buffer conditions was first determined by subjecting known amounts of β-actin cDNA to the RT-PCR procedure, with or without the preliminary exposure to DNase I (Figure 1A). While a typical treatment with DNase I completely abolished any amplification signal, strong amplification was apparent when either DNase I was omitted or the 37°C incubation was not performed. It was therefore apparent that selected DNase I-Mn^{++} incubation conditions effectively allowed excess amounts of target DNA to be digested completely. Thereafter, it was also apparent that DNase I activity was fully denatured by the high temperature of the RT step (compare amplification signals of Figure 1A, lanes b and c), ruling out the possibility that residual activity of this enzyme interfered with RT-PCR steps. However, DNase I might not be as effective on genomic DNA of crude whole-cell lysates as on purified DNA because of the protection against digestion that may be exerted by associated protein. This possibility was evaluated by RT-PCR amplification of HSP70.1 mRNA in pools of two- and four-cell embryos (Figure 1B), taking advantage of the finding mentioned above that four-cell mouse embryos completely lack this message. Results of this experiment showed that the HSP70.1 mRNA amplification signal was dependent only on the number of input two-cell embryos, while addition of increasing numbers of four-cell embryos (i.e., of increasing amounts of an embryo's genomic DNA, but not of HSP70.1 mRNA) had no effect. We therefore conclude that DNase I-Mn^{++} incubation was as equally effective on the crude genomic DNA as it was on the purified one. In this context, it is interesting to note that the presence of Mn^{++} strongly increases DNase I efficiency (2) by allowing this enzyme to digest DNA by double-strand breaks, in con-

trast to the single-strand breaks occurring with Mg^{++} (11). In addition, presence of Mn^{++} was also appropriate for the RT step and thus eliminated the need for a change in buffer ionic composition after DNase I treatment.

A second set of experiments investigated quantitative properties of our RT-PCR procedure. When increasing amounts of β-actin mRNA (transcribed in vitro using T7 RNA Polymerase and the RiboMAX™ Large Scale RNA Production System [Promega]) were subjected to the full procedure, radioactivity incorporation into amplification products was linearly related to mRNA input (Figure 2A). This issue was further investigated with mRNAs of growing oocytes by subjecting decreasing fractions of the same oocyte lysate to RT-PCR amplification with either β-actin or S16 primers (Figure 2B). Results obtained in this experiment were similar to those reported above, radioactivity incorporation into amplification bands being linearly related to initial lysate input with both messages. Finally, the consistency of our method was investigated by RT-PCR of several different lysates of single two-cell embryos (Figure 2C). Similar levels of HSP70.1 and S16 mRNA amplification were achieved in all samples, while the small variation in amplification efficiency that was seldom observed between different tubes (compare Figure 2C, lanes b and c) was normalized by the constancy of the HSP70.1/S16 incorporation ratio.

The last experiment reported in this paper is an example of how our procedure can be applied to a developmental analysis (Figure 3). Quantitative variation of β-actin mRNA was determined in single growing, fully grown and metaphase II mouse oocytes taking the S16 mRNA as internal standard. RT-PCR amplification bands and corresponding β-actin/S16 incorporation ratios clearly indicated that β-actin mRNA underwent a 50% decrease in amount with the end of oocyte growth and then remained constant during oocyte meiotic maturation. This conclusion fully agrees with previous results obtained by Northern blot, an analysis that required a pool of 150–300 oocytes for each determination (1).

The use of rT*th* Reverse Transcriptase for RT-PCR steps gives our method a number of advantages. Among them, the use of the same enzyme for both RT and PCR avoids the need for time-consuming, sequential reactions with different polymerases, as described in previous whole-cell RT-PCR procedures (6,17). The major advantage, however, is that rT*th* Reverse Transcriptase allows the DNase I-Mn^{++} treatment to be directly coupled with the RT step. This in turn eliminates the possibility that RNA recovery is incomplete, as may occur when the lysate is extracted with organic solvents or is passed through a column of bead-immobilized DNase I (17). In fact, our procedure, having the DNase I denaturation step combined with RT, allows the target RNA to be recovered entirely through the procedure and to eventually produce amplification bands that are strictly dependent on initial amounts of target RNA. We conclude that our RT-PCR procedure quantitatively reveals specific message amounts and their relative developmental variation in single mouse oocytes or embryos. This now makes it possible to undertake quantitative transcriptional analyses on individual mammalian oocytes and embryos that have previously been subjected to fine experimental manipulation (such as nuclear/cytoplasmic microinjection or nuclear transplantation) or on single, microsurgery-isolated blastomeres. Thus, it may be helpful in reducing embryo waste in transcriptional analyses of in vitro-fertilized human embryos. If needed, incorporation values obtained with the present procedure can also be expressed as absolute numbers of message molecules that are actually present in the cell. This can easily be achieved by addition to lysate of a known amount of base-mutated/truncated mRNA molecules derived from the message under analysis and subsequent co-amplification of mutated and natural messages with the same primer pair (3). While our method has been designed specifically for studying mammalian oocytes and preimplantation embryos and for analyzing messages coded by intronless genes, its sensitivity and reliability make it also suitable for being used in other systems in which message analysis is hindered by the paucity of biological material.

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