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RNA-Seq profiling of single bovine oocyte transcript abundance and its modulation by cytoplasmic polyadenylation

Juan M Reyes, James L Chitwood, and Pablo J Ross

Department of Animal Science, University of California, Davis

Abstract

Molecular changes occurring during mammalian oocyte maturation are partly regulated by cytoplasmic polyadenylation (CP) and affect oocyte quality, yet the extent of CP activity during oocyte maturation remains unknown. Single bovine oocyte RNA sequencing (RNA-Seq) was performed to examine changes in transcript abundance during in vitro oocyte maturation in cattle. Polyadenylated RNA from individual germinal-vesicle and metaphase-II oocytes was amplified and processed for Illumina sequencing, producing approximately 30 million reads per replicate for each sample type. A total of 10,494 genes were found to be expressed, of which 2,455 were differentially expressed (adjusted $P < 0.05$ and fold change > 2) between stages, with 503 and 1,952 genes respectively increasing and decreasing in abundance. Differentially expressed genes with complete 3'-untranslated-region sequence (279 increasing and 918 decreasing in polyadenylated transcript abundance) were examined for the presence, position, and distribution of motifs mediating CP, revealing enrichment (85%) and lack thereof (18%) in up- and down-regulated genes, respectively. Examination of total and polyadenylated RNA abundance by quantitative PCR validated these RNA-Seq findings. The observed increases in polyadenylated transcript abundance within the RNA-Seq data are likely due to CP, providing novel insight into targeted transcripts and resultant differential gene expression profiles that contribute to oocyte maturation.

Keywords

Oocyte maturation; transcriptome; RNA sequencing; cattle; polyadenylation

Introduction

Oocytes become transcriptionally silent following germinal-vesicle breakdown, so the final stages of oocyte maturation and early embryo development depend on stored transcripts for protein synthesis, which is ultimately regulated by post-transcriptional mechanisms (Paynton et al. 1988; Vassalli et al. 1989). Studies using *Xenopus* oocytes have provided models for translational repression that involve the deadenylation of stored transcripts in germinal-vesicle (GV) oocytes, followed by their activation via cytoplasmic polyadenylation (CP) during oocyte maturation (reviewed by Villalba et al. 2011). Translational repression and activation of such transcripts is mediated by regulatory elements in the 3'-untranslated

region (3'UTR) of mRNAs, including a cytoplasmic polyadenylation element (CPE) and/or a polyadenylation hexanucleotide element (Hex), and their interaction with specific proteins (reviewed by Villalba et al. 2011). Thus, the effects of translational repression and CP provide a metric for changes in the biological needs of the cell since polyadenylation status affects translational activity as well as our ability to measure a transcript's abundance. The use of different reverse transcription priming strategies (e.g. oligo(dT)-based or random primers) allows for changes in the abundance of total (silenced and activated) and polyadenylated (activated) transcript levels to be studied (Nam et al. 2002; Resuehr and Spiess 2003), thereby providing a means to characterize the mechanisms behind transcript management during oocyte maturation.

Microarray-based transcriptome studies in various species have revealed differences in polyadenylated mRNA abundance between the GV and the metaphase-II (MII) stages of human (Assou et al. 2006), mouse (Cui et al. 2007), *Xenopus* (Graindorge et al. 2006), and bovine (Fair et al. 2007; Mamo et al. 2011) oocytes. Some of these differences represent biologically significant changes that contribute to successful oocyte maturation, including the B-type cyclins, which together with cyclin-dependent kinase 1 (*CDK1*; aka *CDC2*) form the metaphase-promoting complex that regulates oocyte maturation in *Xenopus*, zebrafish, and mammals (Choi et al. 1991; Gautier et al. 1990; Kondo et al. 1997). Regulation of B-type cyclin protein levels by CP during oocyte maturation is well-established, and serves to elucidate some of the intricacies of translational repression and CP (reviewed by Mendez and Richter 2001), which have been applied and expanded on to create a *Xenopus*-based predictive tool of CP during oocyte maturation (Pique et al. 2008). Although CPE and Hex are two absolute requirements for CPE-mediated post-transcriptional regulation, pumilio binding element (PBE) is also considered functional since some transcripts with particular motif distribution were shown to be affected by its presence (Pique et al. 2008). Despite the discovery of several essential developmental genes being regulated by CP, there is a need to further elucidate the significant transcripts and pathways involved in oocyte maturation to address issues of mammalian reproductive efficiency.

The advent of RNA sequencing (RNA-Seq) has enabled global transcriptome analysis of single oocytes (Tang et al. 2009) and subsequent identification of differentially expressed genes (DEGs) between different stages. In addition, queries of the 3'UTR of expressed genes for motifs involved in CP enable improved genome annotation, which is particularly poor for UTR sequences (Mangone et al. 2010; Smibert et al. 2012; Ulitsky et al. 2012). In this study, RNA-Seq was employed to identify changes in polyadenylated transcript abundance between single GV and MII bovine oocytes and to determine candidate transcripts subject to CP, with an emphasis on identifying biologically relevant differences.

Results

Single oocyte transcriptome sequencing

Single-oocyte, whole-cell lysates from GV (n=3) and MII (n=3) samples were used as template for cDNA synthesis, with an anchored oligo(dT) primer, and for amplification using a whole-transcript PCR amplification approach. Each stage yielded 21.7 ± 2.8 ng (GV) and 20.9 ± 4.7 ng (MII) of cDNA. Subsequent cDNA sonication, library preparation, and

Illumina sequencing resulted in an average of ~30 million 50-bp reads per oocyte, of which 79.3% mapped to the bovine genome reference (Gene Expression Omnibus accession: GSE61717). The number of reads generated plus the high mapping statistics demonstrated the ability to successfully produce an Illumina library from single bovine cells.

Custom annotation from the reference genome and Illumina reads

Illumina reads from the six oocyte samples were used to expand the bovine genome annotation by inferring transcripts from mapped reads. Potentially novel genes and new transcript variants were identified and added to the Ensembl UMD3.1.68 annotation. The number of genes in the annotation increased from 24,616 to 27,526, and the number of transcripts from 22,750 to 57,019 (annotation file available as a GFF; Supplementary File 1). Of the 19,861 unique protein-coding genes in the original reference, 5,290 underwent extension at the 3' end. The new annotation contained 7,553 known genes with a complete 3'UTR, as determined by the presence of a Hex sequence within the last 30 bp of the transcript. Due to the absence of gene-structure information for novel genes (e.g. the 3'UTR versus coding sequence), 3'UTR analysis was not possible for these genes and were thus excluded from the new annotation. The improved annotation empirically demonstrated the incomplete nature of the original reference genome and provided a means to mitigate some of the deficiencies, thereby enabling more thorough downstream analyses.

RNA-Seq analysis of individual oocytes

In total, 85.6% and 85.3% of the individual sample sequencing reads mapped to the custom annotation, with 71.7% and 72.4% of the reads mapping to gene exon regions for GV and MII oocytes, respectively (Supplementary Table 1). Global transcriptome profiles of single GV and MII oocytes were highly correlated among replicates of the respective sample types (GV, $r = 0.963 \pm 0.01$; MII, $r = 0.974 \pm 0.003$). Heat-map analysis of expressed protein-coding genes ($n=10,494$) enabled accurate hierarchical clustering for samples pertaining to each stage (Fig 1A), collectively demonstrating the reproducibility of single bovine oocyte RNA-Seq.

Among all expressed protein-coding genes, 8,628 were detected (reads per kilobase per million mapped reads (RPKM) >0.4) in both GV and MII oocytes, while 1,553 and 313 were exclusive to GV and MII oocytes, respectively (Fig 1B). Expressed genes included those associated with oocyte maturation and cell-cycle progression (e.g. *CDC25*, *CCNB1*, *WEE2*, *FBXO43*, *MELK*, *CDK1*, and *AURKA*), oocyte-specific genes (e.g. *ZP2*, *ZP3*, *ZP4*, *HIFOO*, *GDF9*, *BMP15*, *STELLA*, and *ZARI*), and CP-related genes (e.g. *CPEB*, *PUM*, *CPSF*, *PAPD4*, *PARN*, and *PABP*) (Table 1, Supplementary Table 2, and Supplementary Table 3). Expression of the aforementioned genes during oocyte maturation supports our current understanding of oocyte biology, given their involvement in essential processes that contribute to successful oocyte maturation and early embryo development.

Differential abundance of polyadenylated transcripts

Statistical analysis of all expressed protein-coding genes revealed 503 and 1,952 genes that significantly increased and decreased in polyadenylated transcript abundance, respectively, from the GV to the MII stage (Fig 1c, Supplementary Table 4). Among the DEG transcripts that increased from GV to MII were those involved in oocyte maturation and cell-cycle

progression (e.g. *CCNB1*, *WEE2*, *FBXO43*, and *MELK*) and CP (e.g. *CPEB4* and *PUM2*) (Table 1, Supplementary Table 2, and Supplementary Table 3).

Functional annotation analysis revealed that genes increasing in polyadenylated transcript abundance were enriched for biological processes that included the cell cycle, macromolecule catabolic process, and cytoskeleton organization (Table 2). For genes that decreased in transcript abundance, generation of precursor metabolites and energy, ribonucleoprotein complex biogenesis, and mitochondrion organization were the most enriched biological processes (Table 3).

3'UTR analysis of DEG transcripts

Of the significantly up- and down-regulated genes, 279 and 918 were found to have a complete 3'UTR (Hex within the last 30 bp), respectively. Most of the up-regulated transcripts (85.7%) but relatively few of the down-regulated transcripts (18.5%) presented with 3'UTR motif combinations involved in CP (Fig 2 and Fig 3). These results suggest that the genes exhibiting increased abundance of polyadenylated transcripts may be predominantly regulated by CP.

Total and polyadenylated transcript abundance

Genes associated with increased (*DNMT3B*, *FBXO43*, *CPEB4*, and *CCNB1*) or decreased (*EZH2*, *GDF9*, *PRDX2*, and *PAIP2*) polyadenylated transcript levels, based on the RNA-Seq analysis, were evaluated by quantitative PCR (qPCR) to determine the polyadenylated (anchored oligo(dT)₂₀ priming) and total (random-hexamer priming) transcript levels in independent GV and MII samples. Seven of the eight genes analyzed demonstrated similarly significant changes in polyadenylated transcript levels between RNA-Seq and qPCR results, demonstrating the validity of RNA-Seq analysis (Fig 4 and Supplementary Table 3); however, *EZH2* showed a decrease in polyadenylated transcript abundance in RNA-Seq but stable expression by qPCR analysis. Most analyzed genes showed no change in total transcript levels between the GV and MII stage (Fig 4), suggesting that polyadenylation changes are primarily responsible for active transcript regulation during oocyte maturation.

Discussion

This study reports single bovine oocyte transcriptome profiling before and after oocyte maturation using RNA-Seq, and examines potential CP activity across the genome. The qPCR analysis conducted on cDNA synthesized using anchored oligo(dT)₂₀ or random primers validated RNA-Seq findings, and suggest that CP is responsible for increased polyadenylated transcript abundance during oocyte maturation.

Transcriptome analysis of single oocytes and/or preimplantation embryos has been reported for a number of species (Tang et al. 2009; Xue et al. 2013; Yan et al. 2013), including cattle (Chitwood et al. 2013; Jiang et al. 2014). Here we expand the number of available datasets exploring the transcriptome of bovine oocytes before and after in vitro maturation using RNA sequencing technology. As in other studies, the paucity of RNA in a single oocyte/embryo required its amplification to obtain quantities sufficient for library preparation, which could potentially introduce biases (Bhargava et al. 2014). Another caveat to our

interpretation is that the MII oocytes used herein were obtained after in vitro oocyte maturation, which may differ from in vivo-matured MII oocytes (Jones et al. 2008; Katz-Jaffe et al. 2009; Kues et al. 2008; Lee et al. 2008; Mamo et al. 2011; Thelie et al. 2007).

Anchored oligo(dT) primers were used in this study to create the sequencing libraries with the intent of determining the levels of polyadenylated transcripts, which could be considered active transcripts (available for immediate translation). Even though a short polyadenine tail (5–10 bp) has been shown to be sufficient for polyadenine-dependent reverse transcription (Graindorge et al. 2006), the length of the polyadenine tail influences the reverse transcriptase efficiency (Gohin et al. 2013), so changes in transcript abundance detected by anchored oligo(dT) priming may represent changes in transcript polyadenylation status.

In agreement with previous studies (Jiang et al. 2014), we found that approximately half of all annotated protein-coding genes are expressed in bovine oocytes. More of these genes were detected in GV- than in MII-stage oocytes, with a large proportion of these genes decreasing in abundance compared to those that increased as maturation proceeded; similar trends were observed in other studies utilizing bovine (Fair et al. 2007; Mamo et al. 2011), human (Assou et al. 2006), and *Xenopus tropicalis* (Graindorge et al. 2006) oocytes. The decrease in transcript levels during oocyte maturation is consistent with the global reduction in total RNA (Bachvarova et al. 1985) and mRNA (Bachvarova et al. 1985; Lequarre et al. 2004; Sagata et al. 1980). Such a decrease in transcript level would be expected if, after onset of maturation, the oocyte is transcriptionally inactive but continues to translate mRNAs produced and stored in the egg during oocyte growth. In addition, a gradual reduction in total polyadenylated mRNA is expected since translated transcripts undergo deadenylation and degradation (reviewed by Evsikov et al. 2006), particularly in the absence of motifs involved in posttranscriptional regulation (Varnum and Wormington 1990). Indeed, the microarray study conducted by Mamo et al. (2011) reported that 1,413 genes (77.5% of DEGs) significantly decreased in transcript abundance during in vitro oocyte maturation, which is similar in number and proportion to what we found in this study (n=1,952; 79.5% of DEGs). A majority (55%) of the DEGs were also in common between the studies, which represents a good level of agreement considering the differences in analysis platform, source of oocytes, maturation protocol, etcetera.

Functional categories represented among the transcripts whose abundance decreases during oocyte maturation included metabolic and energy processes as well as intracellular transport activity (Table 3). These data agree with previous studies (Mamo et al. 2011), and support the biological differences between oocytes before and after maturation. On the other hand, a proportion of DEGs (20%) increased in polyadenylated transcript abundance – including some involved in cell-cycle progression, cytoskeletal organization, and macromolecule catabolism – which are as expected, considering meiosis progresses from the diplotene stage to the MII stage during oocyte maturation (Table 2).

Potential sources for increased transcript abundance include de novo transcription and increased transcript poly-adenine tail length. The presence and level of transcriptional activity during oocyte maturation remains a controversial issue, however. In a previous transcriptome study, 23% of DEGs were significantly up-regulated during oocyte

maturation. Using transcriptional inhibitors and different primer types, the investigators demonstrated that some de novo transcription occurs in bovine oocytes following the resumption of meiosis (Mamo et al. 2011). Even though we observed a similar proportion of up-regulated genes (20% of DEGs), there was very little overlap (6%) in the genes between these two studies; this disparity may be due to detection differences between sequencing-based approaches (RNA-Seq) and intensity-based approaches (microarray). Of note, genes reported to be up-regulated by Mamo et al. (2011), exhibited expression values below our detection threshold (0.4 RPKM) or, if above it, were of low abundance. In contrast, most genes that were confidently demonstrated to decrease in abundance were highly expressed. Another potential explanation for the differences between studies could be the source of oocytes, as GV-oocyte chromatin configuration affects transcriptional state (reviewed by Tan et al. 2009), wherein non-surrounded nucleolus and surrounded nucleolus configurations respectively associated with active transcription and transcriptional quiescence.

Quantitative PCR analysis using different types of primers for reverse transcription supports an important role for CP in polyadenylated transcript abundance. Significant changes in the polyadenylated mRNA fraction (anchored oligo(dT)₂₀-primed) were confirmed by qPCR, whereas no change in total transcript level (random hexamer-primed) was observed for a handful of selected genes. The role of CP during oocyte maturation in *Xenopus*, zebra fish, and multiple mammalian species has been well established (Charlesworth et al. 2013; Tremblay et al. 2005) and shown to be highly conserved (Gebauer and Richter 1996; Gebauer et al. 1994; Tay et al. 2000). In cattle, for example, inhibition of polyadenylation and transcription, but not transcription alone, led to meiotic arrest, thereby supporting the essential contribution of CP to oocyte maturation (Traverso et al. 2005).

The activity of CP in bovine oocytes combined with its known importance during oocyte maturation in other species prompted examination of the expression of CP-associated factors in bovine oocytes. Most genes involved in CP were found to be stably expressed, with some of them changing in polyadenylated transcript abundance during oocyte maturation (Table 2). Cytoplasmic polyadenylation element binding protein 4 (*CPEB4*) increased in polyadenylated transcript abundance during bovine oocyte maturation, suggesting that it may play an important role late in the process of maturation –as reported for *Xenopus*, wherein *CPEB4* replaces *CPEB1* (Igea and Mendez 2010), and for mouse, in which *Cpeb1* and *Cpeb4* translation respectively decreased and increased (Chen et al. 2011). Cleavage and polyadenylation specificity factor 3 (*CPSF3*) was also found to decrease in polyadenylated transcript abundance, as expected given its function in cleaving pre-mRNA in the nucleus. Interestingly, pumilio 2 (*PUM2*), a repressor (reviewed by Miller and Olivas 2011) and activator (Pique et al. 2008) of translation, increased in polyadenylated transcript abundance during bovine oocyte maturation (this study) as did its translation in mouse oocytes (Chen et al. 2011). Mammalian species have no comparable ortholog to maskin; the mammalian *TACC3* gene codes for a protein similar to *Xenopus* maskin, but lacks the EIF4E binding domain (de Moor et al. 2005). Nevertheless, recent findings have pointed towards alternative models of translational repression that are maskin-independent (Cai et al. 2010; Minshall et al. 2007; Tomek and Wollenhaupt 2012), and the expression pattern of CP-associated genes

observed herein is consistent with the activity of such post-transcriptional regulatory mechanisms during bovine oocyte maturation.

Expressed bovine genes with a complete 3'UTR were inspected for the presence, position, and distribution of motifs known to regulate CP in *Xenopus* (Pique et al. 2008) to address potential CP activity within the oocyte. Most genes with increased polyadenylated transcript abundance (85.66%) presented 3'UTR motifs combinations that are associated with CP regulation, whereas relatively few (18.52%) genes that decreased in abundance were categorized as CP-regulated (Fig 3). These results indicate a strong relationship between the presence of a 3'UTR motif and increases in polyadenylated transcript abundance. Our findings are in line with a transcriptome study in bovine GV oocytes that determined that Hex (AATAAA) and CPE (TTTTTATT) motifs are enriched in deadenylated transcripts compared to polyadenylated populations (Gohin et al. 2013). Reports in mouse (Stutz et al. 1998; Tay et al. 2000) and *Xenopus* (de Moor and Richter 1999) have also demonstrated CPE-mediated post-transcriptional regulation through its involvement with translational repression (deadenylation) in GV oocytes with activation (re-adenylation via CP) in MII oocytes. A transcriptome-wide study examining zebrafish and *Xenopus* pre-gastrulation-stage embryos further demonstrated an association between polyadenine tail length and translational efficiency, which was lost when mechanistic changes in translational control occur during gastrulation (Subtelny et al. 2014). Given that maturing oocytes and pre-maternal-to-zygotic transition-stage embryos both utilize stored transcripts for protein production, it is possible that a similar association between polyadenine tail length and translation efficiency also exists in oocytes. Indeed, polysome-RNA profiling conducted in mouse GV and MII oocytes demonstrated that presence of 3'UTR motifs involved in CP significantly affected the translation of an mRNA (Chen et al. 2011).

Aside from building on previous knowledge of DEGs and the enriched biological processes represented by them, the current study provides information of candidate genes regulated by CP during oocyte maturation. These data imply that CP is highly active during bovine oocyte maturation, probably accounting for the majority of observed increases in polyadenylated transcript abundance. Exactly how CP regulates polyadenylated transcript abundance in the oocyte, however, needs to be determined in more detail. In this regard, in silico analysis of the presented data could be used to improve the identification of regulatory elements that influence transcript management during bovine oocyte maturation.

Materials and Methods

Materials were purchased from Sigma-Aldrich Inc. (___), unless stated otherwise.

Oocyte Collection and in vitro maturation

Bovine oocytes were collected from slaughterhouse-derived ovaries, as previously described. Briefly, follicles 2–8 mm in diameter were aspirated using a 21G needle. Cumulus-oocyte complexes (COCs) with more than 5 compact layers of cumulus cells and an even, granulated oocyte cytoplasm were selected and washed multiple times in Hepes-buffered SOF medium (H-SOF). COCs were matured for 24 hours at 38.5°C and 5% CO₂ in M199 medium supplemented with 10% fetal bovine serum (FBS) (HyClone, USA), 0.1 mM

alanyl-glutamine, 0.2 mM sodium pyruvate, 5 $\mu\text{g mL}^{-1}$ gentamicin (Gibco, USA), 50 ng mL^{-1} human epidermal growth factor, 50 ng mL^{-1} ovine follicle-stimulating hormone (FSH; NHPP), 3 $\mu\text{g mL}^{-1}$ bovine luteinizing hormone (LH) (Sioux Biochemical, USA), and 0.1 mM cysteamine hydrochloride.

Oocytes (GV and MII) were denuded from cumulus cells by vortexing for 5 minutes in H-SOF containing 1 mg/mL hyaluronidase. For RNA-Seq, single GV and MII oocytes were placed in a tube with minimal amount of media ($<1 \mu\text{L}$), snap frozen in liquid N_2 , and stored at -80°C . For qPCR analysis, groups of 40 oocytes were placed in 25 μL of PicoPure RNA Extraction Buffer (Applied Biosystems, USA), incubated at 42°C for 30 minutes, and stored at -80°C until processing. Oocyte viability and the stage of meiosis were confirmed immediately before harvesting by staining the oocytes with Hoechst 33342 (10 $\mu\text{g mL}^{-1}$) and imaging under UV light. Oocytes with bright cytoplasmic staining or uneven cytoplasm were discarded.

Library preparation and sequencing

RNA was amplified using the Clontech Smarter Ultra-Low RNA Kit for Illumina sequencing (Clontech, USA). Three single GV (GV14, GV15, GV15c) and MII (MII14, MII14c, MII15) oocytes were thawed and immediately lysed by aspirating them through a small bore glass pipette in 3.5 μL of Clontech Reaction Buffer. The whole oocyte lysate was used for reverse transcription using an anchored oligo(dT)-priming approach, and amplification by PCR as indicated by the kit protocol. The number of PCR cycles used during cDNA amplification is represented in the sample name (14 or 15).

Amplified cDNA was evaluated using a 2100 Bioanalyzer (Agilent Technologies, USA) and fragmented using a Covaris S2 sonicator (Covaris, USA). Fragmented DNA was used to prepare sequencing libraries using the Illumina TruSeq DNA Kit v1 (Illumina, USA), with small modifications. DNA adaptors/indexes were diluted (from 1:10 to 1:50) to account for low cDNA input (ranging from 10.6 to 24.9 ng) and 15 PCR library amplification cycles were conducted. All libraries were sequenced with an Illumina HiSeq 2000 instrument in a single lane.

Custom annotation of the bovine transcriptome

Reads from each sample were trimmed (first 2 bp were removed), combined, filtered for duplicates, and used to extend the Ensemble annotation of the bovine reference genome (Ensembl UMD3.1.68) using the large-gap-mapping and transcript-discovery algorithms within the CLC Genomics Workbench (CLC Bio, Denmark). The custom reference genome generated was used for all downstream analysis.

RNA-Seq data analysis

The six sets of trimmed reads (including duplicates) were individually mapped to the annotated transcripts in the custom bovine reference genome using the RNA-Seq tool in CLC Genomics Workbench, which calculated gene expression levels as RPKM. A gene was considered expressed if its RPKM value was greater than 0.4 in at least two of the three

replicates in a stage (Ramskold et al. 2009). Pearson's Correlations between replicates within sample types were calculated based on RPKM.

Total exon reads were analyzed using the DESeq package in R to determine differential gene expression. The effective library sizes were calculated based on total gene reads. DESeq results were filtered according to statistical significance (adjusted $P < 0.05$) and fold-change (> 2). DAVID Bioinformatics (<http://david.abcc.ncifcrf.gov/tools.jsp>) was used for Gene ontology (GO) biological-process enrichment using the human ortholog gene names along with a human gene background, given the better annotation of gene functions in this species. Heat-map analysis was conducted in R (<http://www.r-project.org/>) using the heatmap.2 function within the gplots package (v.2.11.0), with variance-stabilized data output by the DESeq package v.1.10.1 (Anders and Huber 2010).

3'UTR motif analysis

The 3'UTR sequence of DEGs was retrieved from the custom annotation. For genes with multiple transcripts, the largest UTR that contained a Hex within the last 30 bp was used. The algorithm developed by Pique et al. (2008) was used to determine the potential for CP of a queried gene. This algorithm generates predictions based on the presence, position, and distribution of CPE, Hex, and PBE motifs within the 3'UTR.

Generation of exogenous control RNA

Enhanced GFP cRNA was produced as described by Bettgowda et al. (2006), with minor modifications. PCR amplification of the eGFP plasmid with an altered reverse primer (5'-TTTTTACTCCAGCAGGACCATGTGATCGCGC) produced a 706-bp fragment that was then used as a template for in vitro transcription (Promega Inc., USA) to generate *eGFP* cRNA. Proceeding removal of template DNA with RNase-free DNase (Qiagen, USA), cRNA purification was conducted using the RNA Clean and Concentrator-5 kit (Zymo Research, USA).

RNA extraction, cDNA synthesis, and qPCR analysis

Exogenous control *eGFP* cRNA was added to each sample (4 μL at 250 fg μL^{-1}) before RNA extraction. RNA was extracted using the PicoPure RNA Isolation Kit (Applied Biosystems, USA), including DNase treatment and 22 μL elution volume. The eluate was then divided in two 10- μL aliquots that were used for reverse transcription using anchored oligo(dT)₂₀ or random hexamer primers. SuperScript[®] III Reverse Transcriptase (1 μL) (Invitrogen, USA), RNaseOUT (1 μL) (Invitrogen, USA), and anchored oligo(dT)₂₀ (1 μL at 50 uM) (Integrated DNA Technologies, USA) or random hexamers (1 μL at 50 ng/ μL) (Integrated DNA Technologies, USA) were used for reverse transcription, per manufacturer's protocol, with minor modifications. Reaction conditions were as follows: 25°C for 5 minutes (random hexamers only), 50°C for 60 minutes, and 70°C for 15 minutes. The cDNA was stored at -20°C until utilization for qPCR.

Eight genes were examined for relative total RNA and mRNA abundance using the ABI Prism 7500 Real Time PCR System (Applied Biosystems, USA). Genes, primers, and product sizes are listed in Supplementary Table 5. Primers were designed using Primer

Express (Invitrogen, USA). The 20- μ L reaction volume consisted of forward and reverse primers (0.2 μ L each at 100 μ M) (Integrated DNA Technologies, USA), SSO Fast Master Mix (10 μ L) (Bio-Rad, USA), nuclease-free water (4.6 μ L) (Invitrogen, USA), and cDNA (5 μ L; 0.7 oocyte equivalents). Reaction conditions were: 95°C for 20 seconds, 40 cycles of 95°C for 3 seconds, followed by 60°C for 30 seconds and a melt-curve analysis to ensure product specificity. Target genes and the *eGFP* reference gene were run in duplicate. Relative abundance was calculated using the 2^{-C_t} method (Livak and Schmittgen 2001) and statistically analyzed using the Student's *t*-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CP[E]	cytoplasmic polyadenylation [element]
DEG	differentially expressed gene
GV	germinal vesicle
Hex	polydenylation hexanucleotide element
MII	metaphase II
PBE	Pumilio binding element
qPCR	quantitative PCR
RNA-Seq	RNA sequencing
RPKM	reads per kilobase per million mapped reads
UTR	untranslated region.

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Figure 1. Transcription profiles of GV and MII bovine oocytes

(A) Heat-map and hierarchical clustering of all protein coding expressed genes (RPKM-mapped reads >0.4 in at least two of the three replicates within a stage; n=10,494). (B) Number of expressed genes categorized by stage of expression. (C) Scatterplot (MA plot) of the baseMean of normalized counts versus log₂-fold change for all genes generated by DESeq, with black and red data points representing genes with non-significant and significant ($P<0.05$) changes in gene expression, respectively.

Figure 2. Motifs associated with CP within the *CCNB1*, *FBXO43*, and *PRDX2* 3'UTRs
Graphical representation of presence and location of different CP-associated motifs for selected transcripts that significantly increased (*CCNB1* and *FBXO43*) or decreased (*PRDX2*) in polyadenylation levels during oocyte maturation. Average RPKM-mapped reads for GV- and MII-stage oocytes is provided for each gene. Numbers under each motif indicate the position of motif relative to start of the 3'UTR sequence. CCPE, consensus cytoplasmic polyadenylation element; NCPE, non-consensus cytoplasmic polyadenylation element.

Figure 3. Classification of DEGs based on 3'UTR motif analysis

Number of transcripts predicted to be regulated by CP mechanisms in each group of DEGs based on the presence, position, and distribution of CPE, PBE, and Hex motifs (Pique et al. 2008).

Figure 4. Validation of RNA sequencing and examination of total RNA abundance for selected gene transcripts by qPCR

Relative transcript abundance between GV and MII oocytes in cDNA generated from anchored oligo(dT)₂₀-primed (grey bars) and random hexamer-primed (black bars) oocyte RNA. Synthetic *enhanced GFP* mRNA added to samples before RNA extraction was used as an exogenous control for normalization. Fold change equal to one represents the GV baseline to which the MII group was normalized. Asterisks represent significant ($P < 0.05$) differences between GV and MII oocytes for a gene.

Table 1

Expression levels of cytoplasmic polyadenylation associated genes

Gene Symbol ^a	GV RPKM ^b	MII RPKM ^b	Fold change	P-value ^c
<i>CPEB1</i>	2.3±0.9	1.2±0.5	-1.9	0.635
<i>CPEB2</i>	0.6±0.1	1.5±0.8	2.4	0.515
<i>CPEB3</i>	3.2±0.4	4.1±1.1	1.3	0.685
<i>CPEB4</i>	10.6±0.8	39.4±0.9	3.7	4.9E-04
<i>CPSF1</i>	1.2±0.7	0.6±0.4	-1.8	0.861
<i>CPSF2</i>	31.5±1.1	23.4±6.9	-1.3	0.712
<i>CPSF3</i>	39.4±5.4	9.8±0.5	-4.0	4.6E-04
<i>CPSF3L</i>	0.9±0.5	0.1±0.1	-7.3	0.193
<i>CPSF4</i>	6.4±2.3	6.6±1	1.0	1.000
<i>CPSF6</i>	5.9±1.3	5.8±0.5	-1.0	1.000
<i>CPSF7</i>	0.8±0.1	1.2±0.9	1.6	0.989
<i>PAPD4</i>	23.3±1.6	38.8±4.4	1.7	0.292
<i>PARN</i>	71.9±8.8	60.1±3.9	-1.2	0.878
<i>PUM1</i>	1.2±0.4	1.4±0.6	1.1	0.991
<i>PUM2</i>	8.7±0.6	21.1±2.2	2.4	0.025
<i>SYMPK</i>	0.6±0.4	0.3±0.2	-1.8	0.886
<i>TACC3</i>	126.9±25.7	208.5±36.9	1.6	0.299
<i>PABPC4</i>	6.3±0.3	10.1±0.6	1.6	0.449
<i>PABPC1</i>	54.8±17.3	38.2±13.6	-1.4	0.707
<i>PABPN1</i>	10.5±2.1	11.8±1.6	1.1	0.927
<i>PABPC1L</i>	3.9±0.9	0.2±0.1	-18.7	2.6E-07
<i>PABPC5</i>	0.8±0.4	2.1±0.4	2.7	0.189
<i>PABPN1L</i>	0.7±0.1	0.4±0.1	-1.8	0.897

^a: Human ortholog gene symbol^b: Average mapped reads ± standard error;^c: Adjusted *P*-value (DESeq).

Table 2

Enriched Gene Ontology biological processes for polyadenylated transcripts that increased in abundance during oocyte maturation (n=503; adjusted *P*-value<0.05; fold change>2)

Biological Process	Gene Count	<i>P</i> value	Genes
Cell cycle	50	2.1E-06	<i>RAD51C, E2F8, RBM7, WTAP, LATS1, LATS2, TGFB2, CCNE1, RAD21, TARDBP, MTBP, CCNA2, STAG1, ZW10, CDC7, ESCO1, RBBP4, KIF15, SKP2, UBR2, CDC5L, LIG4, ESCO2, WEE2, UHRF1, SASS6, UHRF2, PPP6C, CEP120, NEK2, HAUS2, NEK1, TIPIN, ITGB1, CENPA, FBXO43, PBRM1, TUBE1, GF11, ERCC6L, PARD6B, PDS5B, PDS5A, CKAP5, ATR, CDC27, SMC3, CCNB1, HDAC3, TP53INP1</i>
Macromolecule catabolism	45	6.4E-05	<i>XIAP, DZIP3, IDE, EDEM1, ZNRF2, NGLY1, USP53, SQSTM1, CASP8, MTBP, FBXO43, FBXO4, RNF34, FBXO45, ADAM9, PAN2, ADAM10, UBE4A, SMG5, SKP2, BIRC6, HACE1, GTF2H3, HERC4, ERLIN1, UBR2, CDC27, WDR48, CCNB1, USP28, DNA2, UHRF1, UHRF2, TRIM33, DCP1A, MGAM, FBXL4, ASB3, APAF1, AMFR, FBXO33, RNF19B, USP25, RNF111, USP26</i>
Mitotic cell cycle	29	6.8E-05	<i>PPP6C, NEK2, NEK1, HAUS2, TIPIN, LATS1, ITGB1, LATS2, CCNE1, RAD21, CENPA, TARDBP, MTBP, PBRM1, GF11, CCNA2, ZW10, STAG1, ERCC6L, CDC7, PDS5B, PDS5A, CKAP5, KIF15, SKP2, CDC27, SMC3, WEE2, CCNB1</i>
Microtubule dynamics	14	8.3E-03	<i>CEP120, NEK2, CKAP5, HAUS2, ASZ1, DOCK7, SMC3, HOOK1, SS18, HDAC3, SASS6, CENPA, TUBE1, ZW10</i>
Cytoskeleton organization	26	9.1E-03	<i>CEP120, CNN3, NEK2, WASF1, HAUS2, FERMT2, ASZ1, ITGB1, HOOK1, KRAS, CENPA, TUBE1, FGD6, DLG1, FGD4, ZW10, FMNL2, CKAP5, NF1, PRKCI, DOCK7, SMC3, SS18, SASS6, HDAC3, CRK</i>

Table 3

Enriched Gene Ontology biological processes for polyadenylated transcripts that decreased in abundance during oocyte maturation (n=1,952; adjusted *P*-value<0.05; fold change>2)

Biological Process	Gene Count	<i>P</i> value	Genes
Energy pathways	84	1.5E-14	<i>LDHC, LDHB, LDHA, ATP6V0E1, OXA1L, UQCRC1, OGDHL, CYC1, NDUFAB1, UQCRCF1, PDHB, NDUFS7, NDUFS5, UQCRC10, IDH3G, UQCRC1, GOT1, FXN, PPP1R1A, ATP5O, PDHA1, NDUFS3, ATP5I, COX17, ATP5H, EDARADD, NDUFB11, NDUFB10, SLC25A4, ALDH5A1, SUCLG1, NDUFC2, NDUFA13, COX4I1, NDUFC1, ATP6V1D, NDUFA12, ATP6V1F, COX6C, ACADVL, TXN, PGM1, ATP5C1, FDX1L, ATP1F1, MDH2, ACAA1, MDH1, NDUFB3, ALDOA, NDUFB5, ECH1, TXN2, NDUFB9, ALDOC, HK1, ATP5G2, ATP5G1, ATP6V0B, ATP6V0C, TPI1, GYS1, C16ORF7, GAPDH, ETFB, ENO1, NDUFA4, NDUFA5, NDUFA2, NDUFA3, NDUFA8, COX7A1, NDUFA9, NDUFA7, FADS3, IDH3B, GYG1, SDHA, SDHB, SDHC, ATP6V1E1, NDUFV2, COX6A1, PGK1</i>
RNA-protein complex biogenesis	46	1.6E-06	<i>EIF6, NCBP1, NHP2L1, LSM6, NUFIP1, SNRPD2, PIN4, FCF1, MINA, UTP11L, PRMT7, RPL7, PRMT5, RPL5, AATF, GEMIN6, IMP4, FTSJ2, GEMIN4, RPS24, EXOSC8, EMG1, SNUPN, EXOSC4, BYSL, EXOSC5, DDX1, RPL26, RPL24, EXOSC1, RPS6, SF3A1, NOP10, RPF1, FBL, TARBP2, RCL1, DDX56, NOP2, PIH1D1, RPS16, RRS1, SNRPC, ALG11, NHP2, TXNL4A</i>
Glycolysis	17	9.2E-04	<i>ALDOA, LDHC, LDHB, LDHA, ALDOC, OGDHL, HK1, PDHB, TPI1, PGM1, PDHA1, PGK1, GAPDH, MDH2, ENO1, EDARADD, MDH1</i>
Intracellular protein transport	61	1.2E-02	<i>SEC24B, CLTA, AP1G2, TIMM17B, AP3S2, SYNGR1, IMM2L, FXC1, TIMM9, VPS16, PRL, SAR1A, TOMM34, TMC06, STX4, SNUPN, SIX3, NDUFA13, VTI1B, TIMM23, IPO4, TOMM22, MRPL45, KPNA2, SEC23B, NXT1, BID, DERL2, AP1M1, AP1M2, COPZ1, TIMM10, SNX1, CALR, PEX19, STX18, TOMM5, SCG5, APBA1, AP2M1, IPO13, STRADA, LIN7B, TOMM40, GTF2A1L, STRADB, GABARAP, YWHAH, RPL23, TOM1L1, COG7, TOMM70A, TSC2, YWHAQ, PTTG1IP, HGS, TRPC4AP, SSR4, GGA2, SSR2, TOB1</i>
Purine ribonucleotide metabolism	29	1.7E-02	<i>ALDOA, ATP6V0E1, ATP1B3, ATP5G2, MYO9B, ATP5G1, ATP12A, ADA, PPAT, ATP6V0B, ATP6V0C, ATP5O, C16ORF7, ATP5I, ATP5H, ATP5J2, AK1, MYH6, GMP5, ATP6V1D, ATP6V1F, NME4, NME5, NME1, ATP6V1E1, ATP5C1, ADLS, PAICS, PRPS1</i>
Cofactor metabolism	35	4.7E-02	<i>COASY, LDHB, PPOX, ALDH1L2, PPCDC, SDHAF1, PDHB, MTHFS, TPI1, IDH3G, FXN, LIAS, LIPT2, PDXX, ALDH5A1, SUCLG1, MTHFSD, IDH3B, COQ9, SOD1, SDHA, BLVRA, HAGH, ISCU, COQ3, SDHB, GGT6, PGLS, SDHC, SLC25A38, UROD, MDH2, MDH1, CRYZL1, ACSM4</i>
Mitochondrion organization	33	5.9E-04	<i>BID, OXA1L, CLU, TIMM17B, TIMM10, MPV17, TIMM50, BCL2L1, SDHAF1, NDUFAF3, NDUFS7, IMM2L, FIS1, NDUFS5, HTRA2, FXN, TOMM5, FXC1, TIMM9, HSPA4, DNAJA3, TOMM34, SLC25A4, SSBP1, TOMM40, BCS1L, PIM2, TIMM23, C10ORF2, TOMM70A, PTCD2, TOMM22, DAP3</i>
tRNA metabolism	26	1.7E-02	<i>RPP38, HSD17B10, PUS3, ELAC2, NARS, LSM6, PUS10, QARS, TRMT1, DTD1, METTL1, ADAT2, THG1L, DUS1L, MARS, RPP25, SARS, AARS1, FBL, URM1, RARS, FARSB, HARS, FARSA, POP5, POP7</i>
Aerobic respiration	12	2.9E-02	<i>SDHA, SDHB, OXA1L, IDH3G, UQCRC1, FXN, SUCLG1, SDHC, IDH3B, PDHB, MDH2, MDH1</i>