Spatiotemporal expression of transcriptional regulators in concert with the maternal-to-embryonic transition during bovine *in vitro* embryogenesis

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Abstract

Cleavage-stage bovine embryos are transcriptionally quiescent until they reach the 8- to 16-cell stage, and thus rely on the reserves provided by the stored maternal mRNAs and proteins found in the oocytes to achieve their first cell divisions. The objective of this study was to characterize the expression and localization of the transcriptional and translational regulators, Y box binding protein 2 (YBX2), TATA box-binding protein (TBP), and activating transcription factor 2 (ATF2), during bovine early embryo development. Germinal vesicle (GV)- and metaphase II (MII)-stage oocytes, as well as 2-, 4-, 8-, 16-cell-stage embryos, morula, and blastocysts, produced *in vitro* were analyzed for temporal and spatial protein expression. Using Q-PCR, ATF2 mRNA expression was shown to remain constant from the GV-stage oocyte to the four-cell embryo, and then decreased through to the blastocyst stage. By contrast, the protein levels of ATF2 remained constant throughout embryo development and were found in both the cytoplasm and the nucleus. Both TBP and YBX2 showed opposite protein expression patterns, as YBX2 protein levels decreased throughout development, while TBP levels increased through to the blastocyst stage. Immunolocalization studies revealed that TBP protein was localized in the nucleus of 8- to 16-cell-stage embryos, whereas the translational regulator YBX2 was exclusively cytoplasmic and disappeared from the 16-cell stage onward. This study shows that YBX2, TBP, and ATF2 are differentially regulated through embryo development, and provides insight into the molecular events occurring during the activation of the bovine genome during embryo development *in vitro*.

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Introduction

In the bovine as in other vertebrate species, the very early embryo is transcriptionally silent and the onset of transcription occurs at a precise stage of development, which is species specific (reviewed, Telford *et al.* 1990). Early embryos divide and subsist by using the stockpile of maternal proteins and mRNAs stored in oocytes during their growth. Before this maternal reserve is exhausted, the embryo starts to produce new mRNAs by activating the transcription of their own genome to assure their development. This mRNA replacement occurs for the majority of the maternal mRNAs and is called the maternal-to-embryonic transition (MET). Even if the majority of factors involved in the MET remain elusive, it is understood that different conditions and mechanisms are required by the embryo to activate the transcription from the newly permissive chromatin (Schultz 2002). The understanding of the mechanisms and factors present at the MET are critical for improving methods and conditions for embryo production as the developmental block is observed at this stage *in vitro*.

According to previous studies, the major embryonic transcriptional activation corresponding to the MET in

bovine occurs around the 8- to 16-cell-stage embryos (Camous *et al.* 1986, King *et al.* 1988, Frei *et al.* 1989, Kopeckny *et al.* 1989, Telford *et al.* 1990). However, subsequent studies have shown that some transcription also occurs in earlier stages, in the two- or four-cell embryos (Barnes & First 1991, Plante *et al.* 1994, Hyttel *et al.* 1996, Viuff *et al.* 1996, Lavoie *et al.* 1997, Memili *et al.* 1998, Natale *et al.* 2000). However, this early transcription is slighter and the major activation of transcription still appears to occur in the eight-cell-stage embryos.

One reason explaining the transcriptional silencing in pre-MET embryos is the presence of an inhibitive state of chromatin in these stages (Newport & Kirschner 1982, Majumder *et al.* 1993, Wiekowski *et al.* 1993). One major modification inducing remodeling of this silent chromatin is the acetylation of the histone tails from the nucleosome, which results in a relaxed chromatin structure, and therefore allows the binding of the transcriptional machinery and transcription activation (reviewed, Hassig & Schreiber 1997). However, even in the presence of a permissive chromatin, the cell needs a functional transcriptional machinery to recruit the RNA polymerase II to the transcription initiation sites.

At least in *Xenopus laevis*, deficient basal transcriptional machinery is a limitative aspect for transcriptional activation pre-MET. Indeed, the injection of exogenous TATA box-binding protein (TBP) in early embryos in permissive conditions results in a premature activation of transcription, prior to the normal timing of MET (Almouzni & Wolffe 1995). TBP is a general transcription factor that has a key role in the RNA polymerase II recruitment, and its absence in *Xenopus* pre-MET embryos (Veenstra *et al.* 1999) confirms the hypothesis that pre-MET embryos are deficient in some key factors necessary for proper transcriptional activity. This regulation of transcription by TBP in early embryos seems evolutionarily conserved since mouse pre-MET embryos show a nuclear presence of TBP, which increases substantially at MET (Worrad *et al.* 1994, Gazdag *et al.* 2007), and the knock down of TBP in zebra fish affects the expression of a subset of genes at the MET (Muller *et al.* 2001).

The maternal contribution of the oocyte in mRNA coding for transcription factors like TBP is crucial to the embryo. These stockpiles of mRNA are masked in messenger ribonucleoprotein complexes (mRNPs) that protect it from premature translation and degradation (reviewed, Weston & Sommerville 2006). Y-box protein 2 (YBX2) is a major component of the mRNPs found in oocytes (Bouvet & Wolffe 1994, Yu *et al.* 2001) and plays an essential role in the storage of maternal mRNA for the normal subsequent embryonic development (Yu *et al.* 2004).

Recent studies have shown that the maternal mRNA levels of stockpiled genes exhibit a rapid decrease in the first cleavage stages in early bovine embryos (Vigneault *et al.* 2004, McGraw *et al.* 2007). However, TBP and YBX2 display an mRNA profile, in which the mRNA levels are nearly constant until the four-cell stage and do not increase significantly at the blastocyst stage (Vigneault *et al.* 2004). This implies that these factors are conserved until their presumptive role in the MET at the 8- to 16-cell stage.

Another transcription factor, activating transcription factor 2 (ATF2), involved in the transcription of a wide selection of genes (reviewed Bhoumik *et al.* 2007), is present and active in MET embryos in *Xenopus*, which propose a transcriptional regulatory role for ATF2 in pluripotent embryos (Villarreal & Richter 1995). However, ATF2 presence and expression in mammalian embryos were never investigated.

The objective of this paper was to reveal the temporal expression and localization patterns of YBX2, TBP, and ATF2 in bovine early development and to establish their relationship with the timing of the MET. These genes were chosen for their known involvement in the MET of other species and for their particular mRNA profile in early embryo development. Besides identifying factors potentially implicated in MET, we also report a particular relationship between mRNA levels and protein expression during bovine pre-MET development.

Results

ATF2 mRNA quantification in bovine early embryo development

Real-time RT-PCR experiments for ATF2 showed an mRNA quantification pattern (Fig. 1) similar to the genes of maternal origin, which is also comparable with the mRNA profiles obtained for YBX2 and TBP in our earlier study (Vigneault *et al.* 2004). The levels of mRNA remained constant from the GV-stage oocyte until the four-cell-stage embryo. At the 8- to 16-cell stage, the levels of ATF2 mRNA decreased and remained low throughout the blastocyst stage.

Immunoblot analysis of YBX2, TBP, and ATF2 throughout bovine embryo development

We sought to determine the relative protein expression of YBX2, TBP, and ATF2 during early developmental stages (oocyte to blastocyst). First, antibody specificity in bovine tissues was demonstrated on immunoblots (Fig. 2). During early embryo development, YBX2 protein levels remained high until the eight-cell stage and decreased considerably after to the point of being barely detectable in the blastocyst stage (Fig. 3A). TBP detection revealed a very different pattern during the same period of development. The TBP protein level detected remained very low in the first stages of development and tended to increase at the mid-eight-cell stage (Fig. 3B). This increase accentuated and became significant in the later stages and progressed to its higher level in the blastocyst stage (Fig. 3B). As for ATF2, it showed a different protein expression pattern as its protein detection level remained invariable during the

Figure 1 Quantification of ATF2 mRNA in bovine oocytes and early embryos using real-time RT-PCR. Each developmental stage was performed in triplicate using one oocyte or one embryo per reaction. The relative mRNA levels shown represent the quantity of transcript corrected with the GFP value obtained for each pool. The highest level in each case was given the arbitrary value of 100 and the levels shown are relative to the highest level (mean $\pm$ s.e.m). GV, GV oocytes; MI, MI oocytes; 2C, 2-cell-stage embryos; 4C, 4-cell-stage embryos; 8C, 8-cell-stage embryos and 16C, 16-cell-stage embryos. Different letters indicate a significant difference ($P < 0.05$).

Figure 2 Immunoblotting showing antibodies specificity with bovine protein samples. (A–C) Specificity of antibodies used for the detection of bovine YBX2, TBP, and ATF2 protein respectively. Thirty and 100 GV oocytes were used for YBX2 and ATF2 respectively, while 80 blastocysts were used for TBP.

stages investigated (Fig. 3C). A steady strong signal was observed for ATF2 in all stages examined, from the GV oocyte to the blastocyst. β -Actin detection was used as a well-loading control to ensure that equal amounts of protein were loaded in each well (data not shown).

Immunolocalization of YBX2, TBP, and ATF2 in oocytes and embryos

Immunofluorescence experiments revealed that YBX2 is present throughout the bovine preimplantation embryo development (Fig. 4). YBX2 was exclusively cytoplasmic in all stages detected and appeared to be preferentially located in the cortex instead of being randomly diffuse in the cytoplasm. Furthermore, as shown in Fig. 4, and immunoblots (Fig. 3), YBX2 was not detected at the same intensity in all stages studied. While a relatively high concentration of the YBX2 protein was detected in the period ranging from the GV oocyte to the eight-cell stage, very low amounts were detected afterward.

The TBP localization pattern in the oocyte and early embryos (Fig. 5) was very different from YBX2. Primarily, in contrast to YBX2, the signal intensity was very low in the GV oocyte and very high in the blastocysts. We detected a faint cytoplasmic presence of TBP protein in all stages examined. In the matured oocyte, TBP seemed associated with the mitotic spindle as it was located around the chromosomes. Subsequently, in the two-, four-, and early eight-cell embryos, a faint nuclear signal for TBP was detected, but the signal in the nucleus intensified in the late eight-cell-stage embryos. This increase in the signal intensity was accentuated in the subsequent stages of development as a very bright nuclear signal was observed in the 16-cell-, morulae- and blastocyst-stage embryos.

Figure 3 Immunoblotting showing protein expression throughout development. (A–C) Developmental expression of YBX2, TBP, and ATF2 in bovine oocytes and early embryos. Protein content of 50 (or 75, see the Materials and Methods section) oocytes and embryos of each stage were loaded in each lane. GV, GV oocytes; MI, MI oocytes; 2C, 2-cell-stage embryos; 4C, 4-cell-stage embryos; 8C, 8-cell-stage embryos; 16C, 16-cell-stage embryos; 32C, 32-cell-stage embryos and Blast., blastocysts. Blots were repeated three times ($n=3$) and a representative blot is displayed. Different letters indicate a significant difference ($P<0.05$).

Figure 4 Immunolocalization of YBX2 in oocyte and early embryo development. Confocal representation of oocytes (GV and MII) and embryos (2-, 4-, early 8-, late 8-, 16-cell, morulae, and blastocyst) stained in green with anti-YBX2 antibody and in red with propidium iodide to visualize the DNA. Magnification 600 $\times$. Scale bar = 100 μ M.

The ATF2 protein signal remained relatively constant in all stages of bovine early embryo development (Fig. 6), which is consistent with the immunoblot results (Fig. 3). In every stage examined, both cytoplasmic and nuclear signals were detected with the exception of the metaphase II oocytes, where the nuclear envelope was absent and ATF2 was exclusively cytoplasmic.

Discussion

Our results reveal the expression and localization of an RNA-binding protein, YBX2, and two transcription factors, TBP and ATF2, in bovine early embryo development. This provides insight into the regulation and the potential involvement of these factors in the MET during bovine embryo development *in vitro*.

Because the bovine embryos are transcriptionally silent until MET that occurs at the 8- to 16-cell stage, various mechanisms have to be present to protect the stocked maternal mRNAs from degradation until their

translation. In mouse embryos, it is recognized that YBX2 performs such functions (Yu *et al.* 2001, 2003). This protein is a member of the Y-box family, which binds mRNAs in order to prevent their degradation and also suppresses their translation (Yu *et al.* 2002). YBX2 mRNA is also present in the bovine and we have previously characterized its expression during early embryo development (Vigneault *et al.* 2004). The present study demonstrates that the protein expression of YBX2 is similar to the mouse, in that it is more abundant in the early cleavage stages, and decreases after MET (Yu *et al.* 2001). Our results also showed a prevalence of YBX2 in the cortex of oocytes and early embryos, as previously showed by Yu *et al.* (2001) in mouse, where it is associated with the cytoskeleton. According to its expression pattern, YBX2 would avoid the premature translation of mRNA in early embryos and keep specific mRNAs intact until they are recruited for specific needs in the developing embryo. Because 16-cell-stage embryos are able to transcribe their own mRNAs, the

Figure 5 Immunolocalization of TBP in oocyte and early embryo development. Confocal representation of oocytes (GV and MII) and embryos (2-, 4-, early 8-, late 8-, 16-cell, morulae, and blastocyst) stained in green with anti-TBP antibody and in red with propidium iodide to visualize the DNA. Magnification 600 $\times$. Scale bar = 100 μ M.

Figure 6 Immunolocalization of ATF2 in oocyte and early embryo development. Confocal representation of oocytes (GV and MII) and embryos (2-, 4-, early 8-, late 8-, 16-cell, morulae, and blastocyst) stained in green with anti-ATF2 antibody and in red with propidium iodide to visualize the DNA. Magnification 600 $\times$. Scale bar=100 μ m.

assistance of RNA-binding proteins like YBX2 is no longer required, and thus offers an explanation to the disappearance of YBX2 protein after MET and implies a role for this protein until the onset of embryonic transcription.

Because high amounts of *TBP* mRNA are present in the bovine GV oocytes (Vigneault *et al.* 2004), it was proposed that it could have a potential role in the activation of transcription in the developing embryo. TBP is a core protein that forms the TFIID complex, with other TBP-associated factors (TAFs), which organizes the initiation of transcription. It binds to the TATA box motif in the promoter regions and recruits the basal transcription factors and RNA polymerase II to initiate gene transcription (reviewed, Thomas & Chiang 2006). In the mouse and *X. laevis*, low levels of TBP protein are detected in fully grown and matured oocytes; however, the levels subsequently increase during development up to the embryonic genome activation (Worrad *et al.* 1994, Veenstra *et al.* 1999, Jallow *et al.* 2004, Yang *et al.* 2006, Gazdag *et al.* 2007). Our results also show a similar pattern of TBP protein expression in bovine early development; where protein levels remain low until the eight-cell stage, and then increase through to the blastocyst stage. Thus, this TBP protein accumulation could occur as a result of the translation of maternal *TBP* mRNA stored in oocytes as observed in mouse embryos (Worrad *et al.* 1994). Further evidence suggests that TBP is implicated in embryonic transcription, since its early translation directly activates the basal transcription of *Xenopus* embryos before MET (Veenstra *et al.* 1999) and its knock down reduces transcription at MET in zebra fish (Muller *et al.* 2001). The translocation of the TBP protein into the nucleus of embryos approaching MET suggests an active role for this protein in transcriptional activation in both the mouse (Worrad *et al.* 1994, Wang *et al.* 2006, Gazdag *et al.* 2007) and *Drosophila* embryos (Wang & Lindquist 1998). The present study also provides

evidence for nuclear localization of TBP protein during bovine embryo development just prior to MET, suggesting its involvement in MET in the bovine species as well. However, a low amount of TBP protein was detected in the nucleus of earlier stage embryos, which could be related to the faint transcription detected in these stages (Barnes & First 1991, Plante *et al.* 1994, Hyttel *et al.* 1996, Viuff *et al.* 1996, Lavoie *et al.* 1997, Memili *et al.* 1998, Natale *et al.* 2000). Therefore, the intensifying signal of TBP in the nucleus of late 8- and 16-cell embryos concomitant with the disappearance of YBX2 provides evidence in support of the major genome activation in the bovine occurring at the 8- to 16-cell stage.

Our findings also characterized the mRNA and protein expression and localization of ATF2, also known as the CRE-binding protein 1 (CRE-BP1), a member of the large ATF/CREB transcription factor family. This protein induces transcription by binding to cyclic AMP CRE motifs present in many promoters. Our results were surprising as ATF2 protein levels remained constant throughout embryo development, while mRNA levels decreased significantly at the eight-cell stage, just prior to MET. A similar discrepancy between mRNA and protein levels in bovine embryos exists for the gene *NLRP5* (Pennetier *et al.* 2006). The half-life of the protein could explain such patterns. Another possible explanation for our results could be that the proteins present in oocytes and early embryos came from the oocyte and would be replaced by ATF2 proteins translated *de novo* in early embryos, which would cause the depletion of ATF2 mRNA observed at MET. Moreover, even if the level of ATF2 mRNA detected in blastocysts was quite low compared with the level found in oocytes, this level was not nil and could be at the source of the ATF2 protein detected in this stage. GV oocytes displayed higher amounts of mRNA, but it must be kept in mind that oocytes need to store very high amounts of mRNA

for the first embryonic stages, which is not the case for the blastocyst embryos. In contrast to our results, a study in *X. laevis* showed a different expression pattern for *ATF2* mRNA and proteins where low amounts were detected in the oocyte and increased at MET (Villarreal & Richter 1995). This inconsistency between the two species could be explained by minor transcription observed in bovine pre-MET embryos and the duration of the pre-MET period. The period from the resumption of meiosis to the MET in the bovine lasts 90 h, whereas in the pre-MET phase in *X. laevis*, the 12 cleavages occur in only 6 h where transcription begins (Kimelman *et al.* 1987). Thus, since this interval is very short in *X. laevis*, it could be considered that these embryos are less reliant on minor transcription before MET. Further studies need to be conducted to understand the role of *ATF2* in early embryo development; however, the presence of high levels of *ATF2* in embryonic cells suggests that this transcription factor could be implicated among other things in the expression of genes involved in proliferation and/or maintenance of pluripotency in early embryos.

The present study clearly demonstrates the discrepancy between mRNA and protein profiles in bovine early embryo development, especially pre-MET. Frequently, gene activity is examined by gene expression; however, although there is a strong relationship between mRNA and protein levels in somatic tissues, the situation is drastically distinct in early embryos. Higher mRNA levels of a specific gene in GV oocytes is not reflective of high protein levels at that stage. For some genes, GV oocytes need to stock large amounts of mRNAs to reach MET several days later, at which time embryos become transcriptionally active. For example, *TBP* is a classic maternally inherited gene that is stored in oocytes as mRNA and later translated in embryos when required. However, *YBX2* demonstrates a significant role in the pre-MET period and in the activation of the embryonic genome as both mRNA and protein are elevated in early developmental stages and disappear together after MET. Finally, a distinct situation applies to *ATF2*, where protein levels remained stable in early embryonic development while *ATF2* mRNA levels decreased around MET.

By examining the protein expression and localization of several factors implicated in MET, this study brings forward molecular evidence that strengthens the hypothesis for the timing of the MET at the 8- to 16-cell stage in bovine *in vitro* embryogenesis. First, expression of the *YBX2* protein is in agreement with the period of maternal mRNA masking in bovine embryogenesis, which is a very important period prior to the MET. Secondly, the transcription regulator *ATF2* becomes visible in the nucleus of early bovine embryos prior to the MET, and thirdly, *TBP* accumulates in the nucleus at an appropriate time during the 8- to 16-cell stage. This study also established for the first time the expression and localization of *ATF2* in early mammalian embryogenesis. Further investigations such as knock-

down experiments using RNA interference could reveal functional details on the implication of these factors, especially *ATF2*, in the genome activation of early embryos.

Furthermore, this study highlights the importance of characterizing both mRNA and protein levels of specific genes during early embryo development in the understanding of the roles of these genes in early embryo development, as mRNA and protein levels are not always related. By focusing on only three genes, three distinct relationships between mRNA and protein levels in pre-MET bovine embryos were demonstrated.

Materials and Methods

Tissue collection and embryo production

Oocyte collection and embryo production in synthetic oviduct fluid (SOF) medium were performed as described earlier (Vigneault *et al.* 2004). In brief, bovine ovaries were collected from a local abattoir and the oocytes were aspirated from healthy 3 to 6 mm follicles. Oocytes with a dark uniform cytoplasm surrounded by at least five layers of cumulus cells that refer to healthy cumulus-oocyte complexes were selected for culture in SOF using our defined protocols. The oocytes were fertilized using frozen semen from a mixture of three different bulls and the embryos were cultured also using our standard protocols in SOF medium supplemented with 0.8% BSA. Bovine oocytes and embryos were denuded and washed three times with PBS before freezing at -80°C for further analysis. Bovine oocytes were collected at the GV (uncultured) and MII stages (24 h *in vitro* matured) for use in further experiments. Embryos for use in immunofluorescence were collected at 30-, 42-, 60-, 90-, and 105-hour post-fertilization (hpf) for 2-, 4-, early 8-, late 8-, and 16-cell-stage embryos respectively. For the selection of the late eight-cell embryos, only those that were at the eight-cell stage at 60 hpf were selected and cultured for another 30 h, and thus collected at 90 hpf. Morulae and blastocysts were collected at days 6 and 8 hpf respectively. RT-PCR and immunoblotting experiments were performed on mid-eight-cell embryos that were collected 72-hpf. Experiments were repeated at least three times for each stage for each protein studied, and at least five specimens of each developmental stage (oocytes or embryos) were observed in each reproduction of the experiment. As a result, at least 15 specimens of each developmental stage were observed for each gene studied.

RNA extraction and reverse transcription

RNA extraction was performed using the Absolutely RNA Microprep kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. One picogram of green fluorescent protein (GFP) *in vitro*-transcribed RNA was added before extraction as a technical external control for RNA extraction and RT. The measured amounts of GFP in each pool at the end of the real-time PCR validate and measure the efficiency of the extraction and RT for each pool extracted, and this quantitative

value is used to correct the levels of the other genes measured in these pools. Three pools of 20 oocytes and embryos of each stage were used for extraction. In brief, the RNA extracted from each pool with the Absolutely RNA Microprep kit was precipitated with isopropanol and linear acrylamide and the pellets were washed with 75% ethanol. These dried pellets were resuspended in water containing oligo-dT primers, heated for 5 min at 65 °C to destabilize RNA secondary structures, and chilled on ice before the addition of the Omniscript Reverse Transcriptase (Qiagen) components. The RT reactions were performed at 37 °C for 2 h. For a detailed procedure, see [Vigneault et al. \(2004\)](#). Oligo-dT primers were used instead of random hexamers to measure the polyadenylated form of the mRNA studied, which are related to the population of mRNA susceptible to be translated in the corresponding protein.

Real-time PCR

The primers for ATF2 amplification were designed using Primer3 web ([Rozen & Skaletsky 2000](#)) and from consensus sequences generally derived from human and mouse sequences from the National Center for Biotechnology Information (NCBI). Primers used are 5'-GTAATCACCCAGG-CACCATC-3' and 5'-GGATTCCTGGAACACTAGGC-3'. An annealing temperature of 58°C was used. For detailed LightCycler procedure and quantification, refer to [Vigneault et al. \(2004\)](#).

Immunoblotting

Immunoblotting was performed on bovine oocytes and embryos with each antibody to ensure their specificity for studies, as well as to examine protein expression across the different stages of development. For the antibody specificity testing, 30 GV-stage oocytes were used for YBX2, 100 GV-stage oocytes were used for ATF2, and 80 blastocysts were used for testing with TBP antibody. For temporal protein expression immunoblots, 75 oocytes and embryos of each stage were directly lysed in 2× SDS-PAGE sample buffer, resolved on standard 10% SDS-PAGE gels, and transferred onto nitrocellulose membranes (Osmonics, Minnetonka, MN, USA) using a semi-dry transfer apparatus following the Tris/CAPS discontinuous buffer protocol from Bio-Rad (Bio-Rad Laboratories). The transfer was performed at 1.5 mAMP/cm² for 45 min at room temperature. Membrane blotting was performed as followed: the membrane was blocked in blocking solution for 90 min at room temperature and then incubated with the first antibody overnight at 4 °C. The membranes were washed three times for 15 min with TBS-Tween (0.05%), and incubated with secondary antibody, goat anti-rabbit IgG (H+L), HRP conjugate (Molecular Probes, Invitrogen), and diluted 1:300 000 in 2% non-fat dry milk in TBS-Tween (0.05%). The membranes were washed three times with TBS-Tween (0.05%) and revealed with ECL Advance Western Blotting Detection kit from Amersham (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Blocking solution contained 5% non-fat dry milk for YBX2 and ATF2, 10% goat serum/3% milk for TBP, and 2% ECL advance blocking reagent for β-actin. For incubations, antibodies were diluted in their corresponding blocking

solutions diluted 1:1 with TBS-T. Antibody dilutions were 1:10 000, 1:500, 1:1000, and 1:1000 for YBX2, ATF2, TBP, and β-actin respectively. ATF2 (#sc-187) and TBP (#sc-204) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), β-Actin (#4967) antibody was bought from Cell Signaling (Danvers, MA, USA), and YBX2 was a kind gift from Dr Richard Schultz from the University of Pennsylvania. The same membrane was used for all four antibodies. To ensure the validity of these results, the complete immunoblotting was repeated two more times with new membranes created with new oocytes (50) and embryos (50) of each stage for each replicate. Therefore, immunoblot experiments were performed three times and equivalent results were obtained with each replicate. Protein expression was quantified with the software ImageJ ([Rasband 1997–2007](#)) developed by the National Institutes of Health, and the relative expression level was expressed with respect to the stage with the higher level detected.

Immunofluorescence

GV- and MII-stage oocytes; 2-, 4-, 8-, and 16-cell embryos, morulae, and blastocysts were attached onto poly-L-lysine-coated coverslips; fixed in 2% paraformaldehyde/PBS for 30 min at room temperature; and then permeabilized in PBS with 0.4% Triton X-100 for 30 min. Samples were washed three times for 15 min before blocking for 2 h at room temperature. Blocking solution contained 5% non-fat dry milk for YBX2 and ATF2 and 10% goat serum/3% milk for TBP. The samples were then incubated with the primary antibodies overnight at 4 °C. The antibodies used for immunofluorescence were the same as those used for immunoblotting and they were diluted in their corresponding blocking solutions diluted 1:1 with TBS-T. The dilutions used were 1:5000, 1:300, and 1:500 for YBX2, ATF2, and TBP respectively. The samples were washed three times for 15 min with TBS-T (0.05%) and were incubated for 45 min in the secondary antibody solution containing the Alexa Fluor 488-conjugated goat anti-rabbit IgG diluted 1:1000 (Invitrogen) in a solution of TBS-T containing 2% non-fat dry milk. Three washes of 15 min each with TBS-T were performed prior to incubation for 10 min in propidium iodide (10 µg/ml) in PBS for nuclear staining. Negative controls were prepared with either no primary antibody or normal rabbit serum (Pierce Biotechnology, Rockford, IL, USA). Experiments were performed in triplicates, and at least five oocytes or embryos were observed each time on a Nikon TE2000 confocal microscope (Nikon, Mississauga, ON, Canada).

Statistical analysis

The levels of mRNA were normalized using the GFP external control, as described previously ([Vigneault et al. 2004](#), [McGraw et al. 2006](#)). Statistically significant differences in the mRNA levels for ATF2 and protein levels for ATF2, YBX2, and TBP between each developmental stage were calculated by ANOVA followed by a Newman–Keuls test. Replicates were included in the statistical model. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$). Data are presented as mean ± s.e.m.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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