Localization of mRNA export factors in early mouse embryos

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Abstract

Background: Mammalian early embryogenesis is characterized by drastic alterations of gene expression patterns due to a course of metabolic and structural changes known as the zygotic gene activation. This crucial period of early development was studied in detail on the molecular but not on the ultrastructural level. Nuclear distribution of some essential components of gene expression machinery including mRNA export factors is poorly known.

Methods: In the present work, the distribution of Y14, Aly and NXF1/TAP and the colocalization of these proteins with actin were studied in mouse embryo nuclei during ZGA using double indirect immunofluorescent microscopy.

Results: Redistribution of studied proteins between the nuclei and cytoplasm as well as within the nucleoplasm occurs during ZGA progress. Colocalization of these proteins with nuclear actin is being intensified while ZGA is carried out. We revealed two types of colocalization zones between actin and mRNA export factors: (i) RNase-sensitive zones in the nucleoplasm and (ii) DNase-sensitive zones in the vicinity of nucleolus precursor bodies. The latter zones increased in number and size after artificial suppression of transcription. We suggest these different areas of colocalization between actin and mRNA export factors correspond to the molecular pools with different functions. Some molecules may be involved in mRNA posttranscriptional metabolism, whereas other molecules disengaged from this process are deposited at the periphery of nucleolar precursor bodies.

Conclusions: Distribution patterns of studied mRNA export factors depend on the transcriptional status of the embryo.

Keywords: cell nucleus, mouse embryos, mRNA export, nuclear actin, zygotic gene activation

Background

The initial steps of mammalian embryogenesis are the time of intensive morphological and functional changes that occur both in the cytoplasm and in the nucleus of blastomeres. The structural reorganization is expressed more dramatically in embryo nuclei, since they are being formed de novo after fertilization. In fact, the nuclei of oocytes or spermatozoids that are highly specialized cells demonstrating a complicated dynamics of chromatin, are either being destroyed at the latest stage of oogenesis [1] or their structure and molecular composition are being reorganized crucially after fertilization [2]. As a result, male and female pronuclei, specific structures of mammalian zygotes, are being formed [3,4]. The pronuclei differ from the nucleus of the germ cell and/or somatic cell by the ultrastructural organization and functional properties.

It was established that the pronuclei are transcriptional silent during the appointed time after fertilization [5,6]. At this time, developmental control is managed by molecules accumulated during the oogenesis. Ultrastructural, biochemical and molecular changes resulting in reactivation of transcription are known as zygotic gene activation (ZGA) or embryonic gene activation. Molecular mechanisms of ZGA have been well characterized in different mammals [6-9]. Structural changes which accompany ZGA are described to a lesser extent. In several papers, the morphology and molecular composition of nucleoli, Cajal bodies (CBs) [10-12] or interchromatin granule clusters [13] have been described in early mammalian embryos. However, nuclear structure and dynamics in blastomeres as pluripotent cells are poorly understood yet.

Molecular interactions between mRNA nascent transcripts and factors controlling their posttranscriptional metabolism and export have clearly been documented for somatic cells. Nuclear export of mRNA is managed
by a multiprotein complex that includes RNA-binding proteins, export adaptors and export receptors. Molecular composition of mRNA export complex and functions of the proteins forming this complex have been described in detail [14–16]. Nuclear export of mRNA is also enhanced by the exon-exon junction complex (EJC) representing a binding platform for mRNA export factors [17]. The EJC is loaded onto mRNA during splicing at a precise point several nucleotides upstream of exon-exon junctions [18]. From the structural point of view, a core-shell model has been proposed for EJCs [19, 20]. Stable association of the EJC with mRNA is provided with the core proteins including Y14 [19], whereas EJC shell proteins including REF/Aly are adaptors providing a link between splicing and export [21]. Aly is also considered to as a component of the TREX complex [22, 23]. This protein belongs to the evolutionary conserved family of hnRNP-like proteins REF and participates in mRNA nuclear export directly interacting with NXF1/TAP [24], an essential mRNA export receptor in mammals [25].

In the present work, nuclear distribution of EJC core protein Y14, the shell protein Aly, and the essential mRNA export factor NXF1/TAP as well as colocalization of these proteins with actin were studied in the nuclei of 1-cell and 2-cell mouse embryo during ZGA. 

Methods
Embryo collection
In the present work, inbred BALB/c mice obtained from the animal nursery “Rappolovo” of the Russian Academy of Medical Sciences were used. Females were induced to ovulate by intraperitoneal injections of 5–10 IU of pregnant mare serum gonadotropin (Folligon, Intervet) followed 44–48 h later by 5–10 IU of human chorionic gonadotropin (hCG) (Chorulon, Intervet). The age of embryos was counted from the time of hCG injection.

The embryos were flushed from oviducts using F10 medium with HEPES buffer (Sigma, Saint Louis, MO). M3 medium with BSA (4 mg/ml) and EDTA (10.8 μM/ml) [26] was used for incubation of embryos in 5% CO2 environment at 37 °C.

Antibodies
The following primary antibodies were used: mouse monoclonals 4C4 against Y14 (Santa Cruz Biotechnology, Cat. no sc-32312; dilution 1:50), 11G5 against Aly/REF (Abcam, Inc., Cat. no ab6141; dilution 1:50), 53H8 against NXF1/TAP (Abcam, Inc., Cat. no ab50609; dilution 1:50), and rabbit polyclonal antibody raised against the C-terminus of actin (Sigma, Inc., Cat. no. A2066; dilution 1:100). The specificity of anti-actin antibody was analyzed using Western immunoblotting [27].

Immunofluorescent/confocal microscopy
Embryos were fixed for 1.0 h in 4 % paraformaldehyde in PBS, and then postfixed overnight in 2% paraformaldehyde at 4 °C. The specimens were washed in PBS, permeabilized for 10 min by 0.5% Triton X-100 in PBS and treated for 10 min by 10 % fetal serum (Gibco, New York, USA) in PBS to prevent nonspecific antibody binding. The incubation in a mixture of first antibodies was carried out overnight in a moist chamber at 4 °C. After rinsing in PBS, the preparations were incubated with fluorochrome-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies for 1.5 h at room temperature. Secondary antibodies were FITC- or Alexa 568-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Molecular Probes). After rinsing in PBS, the preparations were mounted in Vectashield (Vector Laboratories, USA).

To inhibit transcription, some late 2-cell embryos (transcriptional active) were incubated in 5 mg/ml solution of actinomycin D (Sigma) for 3 h. Some samples were treated with 20 μg/ml RNase A (Sigma) or with 0.5 ME/μl DNase (Sigma) for 1.5 h at 37 °C before immunostaining.

Preparations were examined with a Leica TSC SL confocal laser scanning microscope (Heidelberg, Germany) equipped with Argen (488 nm) and Helium–Neon (543 nm) lasers. Confocal images were taken with ×63 (NA 1.32) objective. Merged images were obtained using Leica Confocal Software. Contrast and relative intensities of images were adjusted with Adobe Photoshop.

Results
Male and female pronuclei demonstrated different patterns of actin distribution in 1-cell embryos. Only male pronucleus was labeled with anti-actin antibody, whereas female pronucleus remained unlabeled (Figure 1A, B). The nuclei of blastomeres and the polar body in 2-cell embryos were intensely marked with anti-actin antibody (Figure 1C, D). The mRNA export factors, Aly, Y14, NXF1/TAP, were revealed at all stages studied, but distribution of these factors was different depending on the protein and the age of the embryo (Figure 2).

In first-half of the zygote stage (20–24 h after hCG), anti-Y14 antibody displayed intense cytoplasmic staining (Figure 2A); therefore nuclear borders are not clearly seen. At the late zygote stage, fluorescence of pronuclei increased (Figure 2B), but pronuclei were weakly marked as compared to the cytoplasm. At the late 2-cell stage, bright fluorescence of the nuclei was observed (Figure 2C). At earlier stages, Y14 pattern was rather diffuse while clearly visible fluorescent patches were observed at the late 2-cell stage (Figure 2A–C). Already at the earliest stage, anti-Aly antibody marked the pronuclei but not the cytoplasm (Figure 2D–F). Bright fluorescent nuclear foci appeared in late 2-cell embryos (Figure 2F).

At the early 1-cell stage, NXF1/TAP pattern was similar to Y14. Anti-NXF1 fluorescent signal was faint in pronuclei as compared with the cytoplasm (Figure 2G). In late 1-cell embryos, fluorescence of the cytoplasm disappeared and the signal was visualized in pronuclei only (Figure 2H). Similar pattern retained at the late 2-cell stage (Figure 2I). For NXF1 like Y14 and Aly, bright fluorescent patches were
observed. At early stages these patches were revealed at the NPB periphery (Figure 2G, H). Distribution patterns were similar in male and female pronuclei for all studied proteins.

Double immunofluorescent staining with antibodies against actin and Y14 revealed the areas of signal colocalization distributed diffusely in the nucleoplasm. Moreover, some colocalization areas were observed in association with NPB periphery. The number and size of colocalization areas increased depending on the age of the embryo and reached maximum at the late 2-cell stage (Figure 3A–C, 4A). At the early 1-cell stage, Y14 was newer colocalized with actin. At the late 1-cell stage, the colocalization was observed rarely in male and never in female pronuclei.

Colocalization zones at the NPB periphery were not observed in embryos treated with DNase (Figure 4B), but they retained after RNase treatment (Figure 4C). On the contrary, colocalization zones in the nucleoplasm disappeared in RNase-treated embryos (Figure 4C). After artificial suppression of transcription by actinomycin D, a number of colocalization areas associated with NPBs increased (Figure 4D). Similar pattern of colocalization was observed for pairs Aly−actin (Figure 5A) and NXF1/TAP−actin (Figure 5B) in control late 2-cell embryos. After actinomycin D treatment, colocalization pattern for the pair NXF1 − actin (Figure 5C) changes in a similar manner as it was observed for Y14 − actin (Figure 4D). A number of colocalization zones increases both in the nucleoplasm and at the NPB periphery (Figure 5C). However, these zones are less expressed as a result of fluorescence intensity lowering.

Discussion
ZGA in mice embryos is known to be realized in two stages. The first stage, the so-called minor ZGA, is accomplished at the end of the zygotic stage. The second stage, or major ZGA, occurs during the 2-cell stage [6,8,28]. Hence, the experimental groups of embryos selected for the present work allow analyzing nuclei at different transcriptional states: (i) transcriptionally inert nuclei before ZGA (20–24 h after hCG), (ii) nuclei with low transcriptional activity after
Figure 3 - Double immunofluorescent labeling of Y14 (green) and actin (red) in the 1-cell and 2-cell embryos. Double immunofluorescent staining with antibodies against actin and Y14 revealed nucleoplasmic areas where these proteins are colocalized (arrows). These areas are observed both at the periphery of NPBs and scattered in the nucleoplasm. The colocalization is more significant at the late 2-cell stage. 
line A - Female pronucleus at the late 1-cell stage; 
line B - Male pronucleus at the late 1-cell stage; 
line C - Nucleus of the late 2-cell embryo. 
Scale bar 5 µ.

Figure 4 - Distribution of Y14 (green) and actin (red) in nuclei of the late 2-cell embryos after experimental treatments. Colocalization zones at the NPB periphery are not observed in embryos treated with DNase (B), but are retained after RNase treatment (C). On the contrary, colocalization zones in the nucleoplasm disappear in RNase-treated embryos (C). After artificial suppression of transcription with actinomycin D, a number and size of colocalization areas associated with NPBs increased dramatically (D). Arrows show colocalization zones. 
line A - Control embryo; line B - DNase treated embryo; line C - RNase treated embryo; line D - Actinomycin treated embryo. 
Scale bar 5 µ.

Figure 5 - Double immunofluorescent labeling of mRNA export factors (green) and actin (red) in the late 2-cell embryos. 
In control embryos, colocalization patterns for the pairs Aly–actin and NXF1/TAP–actin resemble Y14–actin colocalization pattern as shown in Fig. 4 A. Small irregular patches where mRNA export factors colocalize with nuclear actin are observed in the nucleoplasm and at the NPB periphery. Number and size of colocalization areas increased after actinomycin D treatment (arrows in 5 C”). 
line A - Distribution of Aly and actin in control embryo; line B - Distribution of NXF1/TAP and actin in control embryo; line C - Distribution of NXF1/TAP and actin in embryo treated with actinomycin D. 
Scale bar 5 µ.
All proteins demonstrated a tendency to cluster when supposed up to the date. Colocalization zones in the nucleoplasm may correspond to the active embryos is not surprising. The RNase-sensitive zones at the periphery of NPBs is more difficult to explain. One can suppose that molecular complexes transiently disengaged from mRNA metabolism may localize to these zones. This assumption is confirmed by the increasing of DNase-sensitive colocalization zones in number and size after artificial suppression of transcription.

Another observation concerns the peculiarities of labeling patterns in male and female pronuclei when anti-actin antibody was applied. Male pronuclei were strongly labeled with antibody against the C-terminus of actin, whereas female pronuclei remained unlabeled. At the present time, it is difficult to explain this fact. One can suppose that the revealed distinctions may reflect different levels of metabolic activity in male and female pronuclei [37,38]. Further studies are required to solve this issue.

Conclusions
Distribution patterns of studied mRNA export factors depend on the transcriptional status of the embryo. In early embryos, localization of both NXF1/TAP and Y14 is mainly cytoplasmic while these proteins are revealed mainly in nuclei after ZGA. Aly is concentrated in nuclei independently on the transcriptional status of embryos. After ZGA, all three proteins have a tendency to form clusters in the nucleoplasm and colocalize with nuclear actin.

List of abbreviations
CB: Cajal body
hCG: human chorionic gonadotropin
NPB: nucleolus precursor body
ZGA: zygotic gene activation

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
Both authors contributed equally to this work.

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