—Review—

Regulation of Zygotic Gene Activation in Mammalian Embryo

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The oocytes are terminally differentiated cells; they express oocyte specific genes, e.g., c-mos and the *zona pellucida* genes. After fertilization, the embryos are totipotent: they can differentiate into all types of cells [1]. This remarkable transformation entails reprogramming of the pattern of gene expression. This reprogramming may require the deletion of the program in maternal and paternal genomes before or just after fertilization, and then the establishment of a new program in the zygotic genome. In the early embryos, some proteins specifically working on the regulation of meiotic cell cycles should not be expressed and ones working uniquely in the regulation of preimplantation development should be expressed. In the new program, the order and timing of the activation of particular genes is precisely regulated during development; some genes are first activated and then the activation of a second set of genes follows. How is this order or timing of gene expression regulated? How do embryos know which genes should be activated first and second?

In this article, I describe the mechanism underlying the timing and basis for zygotic gene activation (ZGA) in mammalian embryos, mainly in mouse embryos. Although the mechanism is poorly understood, some knowledge clarifying it has been accumulated recently and I will introduce it here in relation to the regulation of ZGA.

Termination and Initiation of Transcription in Oocytes and Embryos

In growing oocytes, genes are actively transcribed [2], but the transcriptional activity is decreased to the marginal level when oocytes are full-grown. If any remains, it is at a very low level during meiotic maturation. During this period, the program on the maternal gene expression may be deleted. Following fertilization, early embryos are still transcriptionally silent and the development is regulated by using maternally-derived transcripts [3, 4]. ZGA occurs according to species-specific timing. It is known to occur in two phases: initial activation at a low level and in a second burst. In mouse, ZGA definitely starts in the S-G2 phase in 1-cell embryos. BrUTP incorporation into nascent RNA was first detected as early as in the S-G2 phase in 1-cell embryos in *in vitro* transcription assay [5] and *in vivo* after microinjection of BrUTP [6]. In transgenic mice, the expression of paternally-derived transgene was detected in the G2 phase of 1-cell embryos [7, 8]. The microinjection of reporter gene also showed signs of a transcriptionally active state in 1-cell embryos [9–11]. However, the zygotic transcripts are not involved in the regulation of the first cell cycle, since the treatment with α-amanitin, an inhibitor of RNA polymerase II, does not inhibit the first cleavage [3, 12]. The transcripts from the zygotic genome might not be effectively translated in the 1-cell stage embryos [13]. The burst of ZGA occurs at the late 2-cell stage. Until this stage, the degradation of maternally-derived transcripts has progressed and abrupt increase in the amount of zygote-derived transcripts brings about an extensive reprogramming of the pattern of protein synthesis [14]. The transcripts synthesized in this phase are essential to the second cleavage, since the treatment with α-amanitin arrests the embryos at the 2-cell stage [3, 12]. In other species, although the timing is different, ZGA occurs essentially in the same manner as in mouse; it occurs in two phases and the effects of α-amanitin on the cleavage correspond [15]. The time of ZGA and the transition from maternal to embryonic control of development is well documented in the review of Telford *et al.* [15], although recent precise experiments have revealed that the time of initiation of ZGA is earlier than that described in the review on bovine embryos [16].

In almost all species, embryos are arrested at the

Received: August 1, 1997
Accepted: September 1, 1997

species-specific stage when they are cultured *in vitro* [16, 17]. It is interesting that these stages are the same as the ones at which embryos are arrested by treatment with α-amanitin; the development of embryos is arrested in *in vitro* culture at the stage when the transition from maternal to zygotic regulation of the development takes place.

In the following section, I will describe the mechanism by which ZGA is initiated, or the trigger of ZGA, and then the mechanism for regulating specific gene expression.

Trigger of ZGA

In the early '80s, an elegant hypothesis to elucidate the mechanism for the initiation of RNA polymerase I dependent transcription was presented for *Xenopus laevis* embryo by Newport and Kirschner [18]. It was a histone titration model. In *Xenopus*, unfertilized oocytes contain a large maternal stockpile of histone, which is sufficient to support the formation of 15,000–20,000 nuclei. Although the embryos already possess a functional transcriptional apparatus after fertilization, template DNA is tightly compacted into the chromosome with histones and transcription factors cannot gain access to their target DNA. Once the amount of DNA is exponentially increased after 12 rounds of replication, the maternally-derived histones are titrated. The transcription factors now gain access to their target sites on DNA and transcription starts. When DNA content was artificially increased by polyspermic fertilization or microinjection of exogenous DNA, ZGA occurred earlier. This hypothesis was also confirmed for the activation of RNA polymerase II dependent transcription [19, 20], although it is now under discussion whether or not protein synthesis is required for ZGA in addition to the increase in the amount of DNA [21].

In contrast to *Xenopus* embryos, the increase in the amount of DNA is not involved in the initiation of gene activation in mouse embryos. The inhibition of DNA synthesis by the treatment with aphidicolin does not prevent the initiation of transcription. The embryos arrested at the 1-cell stage by the treatment with aphidicolin increased their transcriptional activity to a level comparable to that of the control embryos when they were cultured until the time chronologically corresponding to the 2-cell stage [5]. Furthermore, the increase in the amount of DNA caused by polyspermic fertilization did not increase the transcriptional activity in 1-cell embryos [5]. In contrast to *Xenopus*, the maternal histone pool in 1-cell embryos seems to be sufficient for only 1-2 rounds of DNA replication [22] and hence insufficient to prevent the transcription factors from gaining access to their target sites on DNA.

In mouse, the transcription factors seem to be rate-limiting in ZGA. In 1-cell embryos, the transcriptional activity is much higher in the male pronucleus than in the female one [5, 9], and the concentrations of TBP and Sp1, basal transcription factors, are also higher in the male [23]. This suggests that transcription factors are not sufficient and that the competition for sequestering them occurs between male and female pronuclei during their formation. The male has an advantage over the female since the transcription factors get an opportunity to gain access to DNA preferentially during the exchange of sperm-inherited protamines for maternally-derived histones in the male pronucleus [24]. Indeed, the total transcriptional activity is similar in parthenogenetically activated eggs and normally fertilized eggs [5]. In parthenoactivated eggs, the female pronuclei would be able to sequester the transcription factors efficiently, since they do not experience competition in the absence of male pronuclei. In addition, the total transcriptional activity in dispermic eggs is also similar to the one in normal monospermic eggs, although the DNA content in dispermic eggs is 1.5 times that in monospermic eggs. These results suggest that the transcription factors are rate-limiting; if the transcription factors are not rate-limiting, an increase in the transcriptional activity as a function of an increase in the DNA content would have been observed.

Taken together, the following hypothesis elucidating the mechanism for the initiation of ZGA could be built. Unfertilized oocytes would not possess a functional transcriptional machinery in mice unlike *Xenopus*. Transcription factors would not be synthesized in unfertilized oocytes. Following fertilization, the synthesis of transcription factors starts and brings about the initiation of the transcription from the zygotic genome. Since these transcription factors should be synthesized from maternal mRNA, the mechanism by which some particular mRNAs are translated only after fertilization is required. The mobilization of maternal mRNA would be involved in this mechanism. The elongation of the poly-A tail is known to increase the rate of translation and it seems to occur in mouse 1-cell embryos. The poly-A tail of an unidentified gene transcript is elongated soon after fertilization [25].

Supporting this hypothesis is the fact that the nuclear concentrations of basal transcription factors, TBP and Sp1, are increased during the first and second cell cycles, and that the increases are prevented by treat-

ment with cycloheximide [23]. The inhibition of protein synthesis with cycloheximide prevents 1-cell embryos from initiating gene activation [26, 27]. Nevertheless, it is not possible to exclude the possibility that unfertilized oocytes possess a functional transcriptional apparatus but some labile transcription factors are depleted by their own turnover when protein synthesis is inhibited. It is also possible that some factors, other than transcription factors, which are essential for the regulation of gene activation, are synthesized following fertilization.

In rabbits, however, a factor or factors inhibiting the transcriptional activity may be involved in the regulation of the initiation of ZGA. When the nucleus from the 32-cell stage embryo actively transcribing zygotic genes was transplanted into an enucleated unfertilized oocyte, the transcriptional activity was decreased quickly and then the activity was resumed when the reconstituted embryos reached the stage at which normal embryos starts zygotic gene expression [28]. This is also observed in porcine embryos [29]. These results suggest that there is a factor or factors inhibiting the transcription in the unfertilized oocytes and that such a inhibitory activity is decreased in the course of development, although the possibility cannot be excluded that the transcription factors accompanying the transplanted nucleus were diffused into the cytoplasm and diluted because the amounts of transcription factors are deficient in the unfertilized oocytes. In mice, unfertilized oocytes appear to contain the substance(s) involved in deletion of the maternal program on gene expression [30]. It would be interesting if the factor inhibiting the transcriptional activity also is such a substance.

In Drosophila, the lengthening of the interphase is involved in the regulation of ZGA. Drosophila embryos start their development with a series of short cell cycles (each 10 min long), which have no gap (G) phase. During this period, zygotic genes are transcriptionally silent. After the ninth division, the cell cycle has a G2 phase and becomes gradually longer until cycle 13, which lasts 25 min. Transcription of some genes is detectable during this period, but the transcriptional activity is still low. In cycle 14, the interphase becomes so long that it takes about 1 h to complete the cell cycle. A large increase in the transcriptional activity occurs in this cycle, and especially during the G2 phase maximal activation of most classes of genes is achieved [31–34]. The increase in the activity could be precociously induced in cycle 11 by extending the interphase with cycloheximide [34]. In mammals, however, the lengthening of the interphase does not seem to be involved in the regulation of ZGA. The G2 phase in the first cell cycle in mouse embryos is already longer than that in cycle 14 in Drosophila [35, 36]. In contrast to Drosophila, the treatment with cycloheximide decreases the transcriptional activity in 1- and 2-cell mouse embryos [26, 27]. Although the mechanism for the initiation of ZGA therefore seems to be thus different in insects and mammals, it is interesting that a large increase in the transcriptional activity occurs at cycle 14 at which the G2 phase is lengthened in Drosophila embryos. In the mouse, a large increase in the transcriptional activity occurs at the 2-cell stage in which the G2 phase is lengthened [35, 36]. Furthermore, the treatment with α-amanitin arrests embryos at the 2-cell stage and cycle 14 in mouse and Drosophila [3, 12, 33], respectively.

Regulation of Specificity for Gene Expression

In somatic cells, genomic DNA is compacted in chromatin with nuclear histones, and their expressions are repressed [Reviewed in 37–39]. The expression of specific genes requires gene specific transcription factors as well as basal transcription factors and/or changes in chromatin structure to help transcription factors to gain access to their target sequences of DNA. Immediately after fertilization, genomic DNA should be repressed to establish the regulatory mechanism for gene specific expression in the preimplantation embryos. Gene specific transcription factors and/or changes in chromatin structure are then required for the expression of specific genes to relieve repression.

A transcriptionally repressive state seems to develop after the first mitosis in mouse embryos. The *HSP 70.1* gene is constitutively expressed early in the 2-cell stage without stimulation with heat shock, but the level of expression is greatly decreased before the completion of the second round of DNA replication [8]. This suggests that repression of the *HSP 70.1* gene occurs during the S phase in the 2-cell stage. DNA replication itself is likely to be involved in this repression, since the expression of the *HSP 70.1* gene continued to increase in the embryos in which DNA replication was inhibited by the treatment with aphidicolin, which arrested the embryos at the 2-cell stage, until the time chronologically corresponding to the 4-cell stage [8]. In *Xenopus* oocytes, microinjected DNA is repressed only when it is single-stranded and subject to DNA replication; double-stranded DNA is not replicated and can be actively transcribed [40].

The experiments with the embryos that were microinjected with reporter gene also showed that a

transcriptionally repressive state develops during the 2-cell stage. A reporter gene linked to a promoter, but not to an enhancer, was microinjected into the nucleus to examine the basal transcriptional activity. Whereas the reporter gene microinjected into a male pronucleus of a 1-cell embryo was actively transcribed, the gene microinjected into a female pronucleus or a nucleus of a 2-cell embryo was not efficiently transcribed [41–43]. A possible rationale for these results is that the female pronuclei contains a factor or factors involved in the repression, but that male pronuclei inherently lack such a factor or lose it during the exchange of protamines for histones after fertilization. After the first mitosis, the components of male and female nuclei are unified, and the chromatin structure is remodeled to establish the repressed state.

Active transcription from the repressed gene requires stimulation with activator proteins that bind to promoters or enhancers, and/or remodeling of the chromatin structure. There are two recently highlighted mechanisms for remodeling the chromatin structure. One is the destabilization of chromatin by members of the SWI2/SNF2 family. The SWI/SNF proteins, which are components of RNA polymerase II holoenzyme, can destabilize chromatin and facilitate TBP binding to promoters [44, 45]. The other mechanism is acetylation of histone H4. There is ample evidence that acetylation of histone H4 is involved in the regulation of transcription. The N-terminal domain of histone H4 is involved in the stabilization of the chromatin structure [46, 47]. Acetylation of N-terminal lysines destabilizes it and facilitates the association of transcription factors with chromosome DNA [47, 48]. Histones are hyperacetylated in the active chromatin domain [49] and hypoacetylated in the transcriptionally silent domain [50, 51]. Furthermore, characterization of a histone acetyltransferase and deacetylase revealed that they were a known transcription factor and a gene specific repressor of transcription, respectively [52, 53], which showed that histone acetylation is involved in the regulation of specific gene activation.

There is some evidence suggesting that histone acetylation is involved in the regulation of ZGA, although there is no information on the SWI2/SNF2 family in the preimplantation embryos. First, localization of acetylated histone is changed in the nucleus during the 1- and 2-cell stages [54–56]. Second, the overall acetylation level of histone H4 is increased between the 1- and 2-cell stages (F. Aoki and RM. Schultz, unpublished data). Last, repression can be relieved by increasing the acetylation level of histones in 2-cell embryos. As mentioned above, plasmid DNA microinjected into male pronucleus of 1-cell embryo was actively transcribed, but not into the nucleus of the 2-cell embryo. When 2-cell embryos were treated with butyrate, an inhibitor of histone deacetylase, the expression of the reporter gene was not repressed and the transcriptional activity was increased to a level similar to that in the male pronucleus in the cleavage arrested embryo with aphidicolin, where repression does not occur [41, 42]. Increasing acetylation also protects the endogenous genes from repression. Translation initiation factor eIF-4C is transiently expressed during the 2-cell stage and its expression is repressed at the 4-cell stage. The embryos treated with trapoxin, a potent inhibitor of histone deacetylase, continued to express it [57]. Furthermore, the treatment with trapoxin increased the transcriptional activity assayed *in vitro* at the 2-cell stage, but not the 1-cell stage [5]. These results of experiments also support the idea that a transcriptionally repressive state is developed during the 2- to 4-cell stage and that it is mediated by the changes in chromatin structure.

Enhancers play an important role in the regulation of ZGA. The role of enhancers is to relieve the repression of promoters in the preimplantation embryos [58]. The repression of the reporter gene microinjected into the nucleus of the 2-cell embryo could be relieved by linking the reporter gene to an enhancer. The level of expression of the construct linked to the enhancer was almost the same as that of the enhancerless construct microinjected into 2-cell embryos which were treated with butyrate, in which histone H4 is hyperacetylated and repression was relieved [42]. But enhancers did not stimulate the reporter gene in the male pronucleus of the 1-cell embryo, in which a transcriptionally repressive state had not yet been developed [42, 59]. Requirement for the enhancer is therefore developed during the 2-cell stage. After a transcriptionally repressive state is established, gene specific transcription factors bind to enhancers to relieve repression and activate the specific genes.

It is possible that global genes are activated in the first phase of ZGA before the repressive state is developed. In the first phase, almost all the genes might be activated, leading to the expression of rarely detectable genes. Indeed, 14 of 20 clones specific for the 2-cell stage, obtained from the 2-cell cDNA library subtracted from the 8-cell one, were novel genes [60]. Furthermore, the casein gene which is expressed specifically in mammary epitherial cells only during the lactating stage is expressed transiently in the 2- and 4-cell stages in mouse embryos [61].

Early this year, people were surprised at the news of the production of a cloned animal [62]. This work is valuable due to its contribution to knowledge of the regulation of gene expression as well as its possible industrial application. The cloned animal was produced by transferring the nuclei from mammary epithelial cells into enucleated unfertilized oocytes. The mammary gland is an adult tissue and the epithelial cells are differentiated. After transplantation of the nucleus, the pattern of gene expression should be reprogrammed to make the reconstituted oocyte totipotent. To understand the key to the success of this work, we should know the mechanism by which the pattern of gene expression was reprogrammed in the reconstituted oocyte. It would be helpful in understanding it to know how oocytes acquire totipotency and embryos start their gene expression during normal development.

Conclusion

The following mechanism may regulate ZGA in mouse embryos. After fertilization, chromosome DNA is transiently transcriptionally active in the male pronucleus in 1-cell embryos, although it is repressed in the female one. However, the nuclear concentrations of basal transcription factors are not sufficient to support active gene expression early in the 1-cell stage. During the first and the early second cell cycles, the concentrations of transcription factors are increased, probably by their *de novo* synthesis, and gene expression starts. Until the early or mid 2-cell stage, since the promoters of genes are not repressed, enhancers are not required for specific gene expression, which might cause the global gene expression. The level of expression is, however, low, since the activity of basal transcription factors is still low. The specific gene expression stimulated with enhancers would start after a transcriptionally repressive state is developed during the 2- to 4-cell stage.

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