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Uncoupling of transcription and translation during zygotic gene activation in the mouse

Jean-Yves Nothias[1], Miriam Miranda and Melvin L.DePamphilis[2,3]

Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA

[1]Present address: Institut Alfred Fessard–CNRS, Avenue de la Terrasse, 91 198 Gif-sur-Yvette, France
[2]Present address: National Institute of Child Health and Human Development, Building 6, Room 416, National Institutes of Health, Bethesda, MD 20892-2753, USA

[3]Corresponding author

Zygotic gene expression in mice is delayed by a time-dependent mechanism until the two-cell stage in development. To investigate the basis of this 'zygotic clock', the firefly luciferase gene was injected into mouse embryos, and quantitative assays were used to monitor luciferase gene transcription and translation in individual embryos from single mothers. These studies confirmed, at the mRNA level, previous conclusions about the relative capacities of paternal and maternal pronuclei to transcribe genes, and the requirements for promoters and enhancers during zygotic gene activation. Furthermore, these studies revealed that fertilized mouse eggs can delay expression of zygotic genes by uncoupling translation from transcription. An RNA polymerase II-dependent gene could be transcribed prior to cell cleavage, but it was not translated until zygotic gene expression began (a delay of up to 15 h after injection). The time course for nascent mRNA accumulation was biphasic, with the second phase occurring during zygotic gene expression. If the luciferase gene was injected after zygotic gene expression had begun, then translation was tightly linked to transcription. If the second phase of mRNA accumulation was repressed, then luciferase was not produced. Therefore, translation was linked to the accumulation of mRNA during the onset of zygotic gene expression. Similar biphasic time courses also were observed for RNA polymerase I- and III-dependent transcription. These and other results reveal that the zygotic clock regulates the onset of both transcription and translation of zygotic genes.

Keywords: RNA polymerase/transcription/translation/uncoupling/zygotic gene activation

Introduction

Studies on a variety of animals have revealed that transcription of the haploid genome in oocytes stops when they mature into unfertilized eggs. While fertilization eventually results in activation of zygotic gene expression, the extent of development as well as the time that elapses can vary considerably among different animal species (Yasuda and Schubiger, 1992). For example, before zygotic genes are activated in flies, the fertilized egg has undergone 10 nuclear divisions in 1.5 h, those from frogs have undergone 11 cell cleavages in 6 h, while those from mice have undergone only one cleavage event in 24 h. These differences reflect the capacity of the maternal genome to provide proteins and mRNAs that support the initial stages in development, and to determine when activation of zygotic genes occurs. Even among mammals, the extent of development prior to zygotic gene activation varies among species from one to four cleavage events (Schultz and Heyner, 1992; Schultz, 1993). Thus, the transition from dependence on maternal genes to dependence on zygotic genes constitutes a primary checkpoint in the developmental program of metazoan animals.

RNA synthesis in mouse oocytes diminishes significantly during the latter stages of their growth and declines after the onset of meiotic maturation to levels barely detectable (Kinloch and Wassarman, 1993). By the time mouse oocytes have completed meiotic maturation to form unfertilized eggs, transcription has stopped and translation of mRNA is reduced (Paynton and Bachvarova, 1994). Fertilization triggers completion of meiosis and formation of a one-cell embryo containing a haploid paternal pronucleus derived from the sperm and a haploid maternal pronucleus derived from the oocyte. Each pronucleus then undergoes DNA replication before entering the first mitosis to produce a two-cell embryo containing two diploid 'zygotic' nuclei, each with a set of paternal and a set of maternal chromosomes.

Formation of a two-cell mouse embryo marks the beginning of zygotic gene expression and the transition from maternal to zygotic gene dependence (outlined in Figure 1). Maternal mRNA degradation is triggered by meiotic maturation and is ~90% completed in two-cell embryos, although maternal protein synthesis continues into the eight-cell stage (Piko and Clegg, 1982; Paynton *et al.*, 1988; Schultz, 1993). Transcription has been detected in late one-cell mouse embryos (Clegg and Piko, 1983; Vasseur *et al.*, 1985; Matsumoto *et al.*, 1994; Temeles *et al.*, 1994; Bouniol *et al.*, 1995; Christians *et al.*, 1995), although zygotic gene activation (ZGA) is not evident until 2–4 h after completion of the first mitosis when transcription-dependent protein synthesis begins (Flach *et al.*, 1982; Bolton *et al.*, 1984; Latham *et al.*, 1991; Wiekowski *et al.*, 1991; Schultz, 1993; Christians *et al.*, 1995). Synthesis of zygotic proteins increases 8–10 h later during G_2 phase (Latham *et al.*, 1991), suggesting that transcription of zygotic genes by RNA polymerase II occurs in two phases, an early phase that is restricted to two-cell embryos and a much stronger late phase that is required for further development (Bolton *et al.*, 1984; Latham *et al.*, 1991; Christians *et al.*, 1995).

Although less well characterized, RNA polymerase

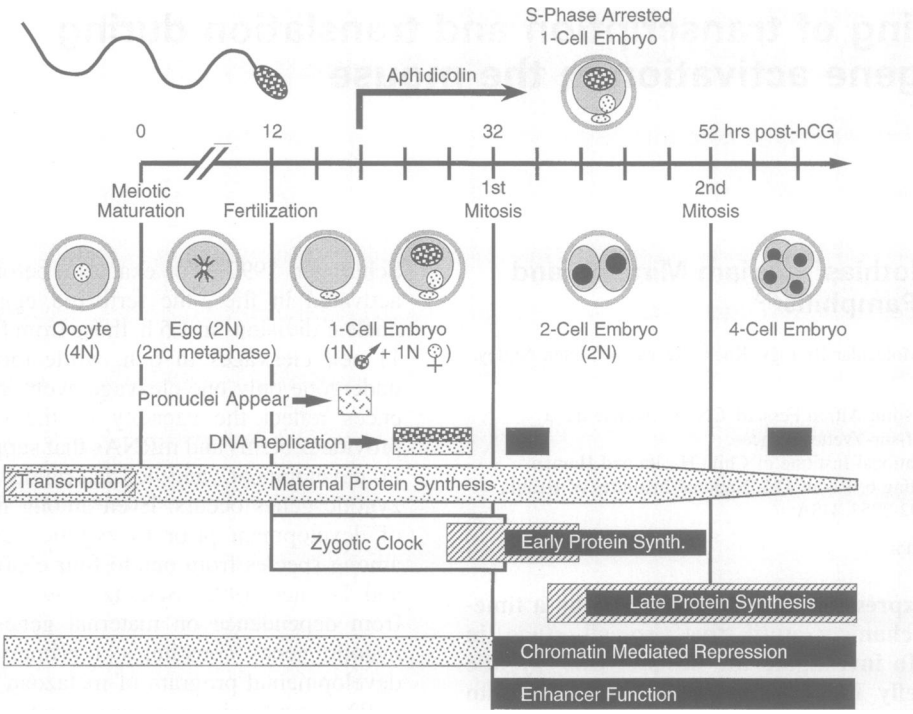

Fig. 1. Events at the beginning of mouse development. Pronuclei appeared between 18 and 21 h after female mice were injected with human chorionic gonadotropin (hCG), a hormone that induces ovulation. Addition of aphidicolin to one-cell embryos prior to the beginning of S phase arrests morphological development, but does not prevent the 'zygotic clock' from activating 'early protein synthesis' or expression of injected plasmid-encoded genes. Except for transcription (▨), events associated with the paternal pronucleus are indicated by ▩, the maternal pronucleus by ⬚ and the zygotic nuclei by ■. Chromatin-mediated repression is evident when promoters are injected into the maternal nucleus of oocytes, the maternal pronucleus of activated or fertilized eggs and the zygotic nuclei of two-cell embryos. The ability to utilize enhancers does not appear until formation of a two-cell embryo.

I- and III-dependent gene expression also begins at the two-cell stage in mouse development. Small nuclear RNAs, whose synthesis depends on RNA polymerase III, begin to accumulate at the two-cell stage in mouse development (Dean *et al.*, 1989). Changes in nucleolar morphology that provide a sensitive indicator of RNA polymerase I-dependent ribosomal gene expression (Kopecny *et al.*, 1989) are localized to the middle of the two-cell stage during mouse development (Geuskens and Alexandre, 1984; Takeuchi and Takeuchi, 1986), and the earliest time at which rRNA synthesis has been detected is the mid- to late two-cell stage (Piko and Clegg, 1982). These results are consistent with the hypothesis that the high rate of ribosome assembly that begins in two-cell embryos requires the coordinate expression of RNA polymerase I-dependent rRNA synthesis and RNA polymerase II-dependent expression of ribosomal protein genes (Taylor and Piko, 1992).

One of the most striking features of zygotic gene expression is that its onset is delayed by a time-dependent mechanism, referred to as the zygotic clock, rather than by a particular cell cycle event. It begins in mouse eggs ~24 h after fertilization, regardless of whether or not the one-cell embryo has completed S phase and formed a two-cell embryo (Bolton *et al.*, 1984; Martínez-Salas *et al.*, 1989; Wiekowski *et al.*, 1991; Schultz, 1993). In contrast, the late phase of zygotic gene expression does not occur without formation of a two-cell embryo (Howlett, 1986). Thus, when one-cell embryos that have not yet formed pronuclei are incubated in aphidicolin, a specific inhibitor of replicative DNA polymerases, development is arrested as they enter S phase, but zygotic gene expression still begins at the time when they would have become two-cell embryos (Figure 1).

In the present study, plasmid DNA encoding the firefly luciferase gene was injected into the nuclei of one- and two-cell mouse embryos in order to determine when fertilized mouse eggs become competent to transcribe and translate genes. Previous studies have shown that luciferase enzyme activity can be measured quantitatively in single embryos, and that expression of this gene requires an embryo-responsive promoter in one-cell embryos and both a promoter and enhancer when in two-cell embryos. Luciferase activity appears only when the injected cell expresses its own genes (Majumder and DePamphilis, 1995; Nothias *et al.*, 1995; this study). For this study, a quantitative method for measuring luciferase mRNA in single mouse embryos was developed so that the appearance of luciferase enyzme activity could be related to luciferase mRNA production.

These studies confirmed, at the mRNA level, previous conclusions about the relative capacities of paternal and maternal pronuclei to transcribe genes and the requirements for promoters and enhancers during zygotic gene activation. In addition, these studies demonstrated that fertilized eggs were capable of transcribing RNA polymerase I (pol I)-, pol II- and pol III-dependent genes prior to undergoing cell cleavage. Surprisingly, however, translation of nascent pol II mRNA transcripts was delayed until a second accumulation of mRNA occurred at the two-cell stage in mouse development, concurrent with the late phase of zygotic gene expression. When this second

phase of mRNA accumulation was repressed, translation did not occur. Therefore, fertilized mouse eggs can uncouple translation of nascent mRNA from transcription of genes, revealing a fail-safe mechanism for delaying zygotic gene expression until formation of a two-cell embryo.

Results

Biphasic transcription of RNA polymerase II-dependent genes in fertilized eggs

Polyomavirus replicates in virtually all cell types and at all stages of mouse development. Moreover, the F101 polyomavirus enhancer has been identified as the most effective enhancer in stimulating the activity of promoters injected into two-cell mouse embryos (Martínez-Salas *et al.*, 1989; Mélin *et al.*, 1993). Therefore, plasmid DNA encoding the firefly luciferase gene (*luc*) driven by the polyomavirus early gene promoter (pPyluc) or the polyomavirus promoter linked to the F101 polyomavirus enhancer (pF101Pyluc) was used to search for a transcriptionally active state in fertilized mouse eggs. DNA was injected into the paternal pronucleus of one-cell mouse embryos that were arrested as they enter S phase by the presence of aphidicolin in their culture medium. More than 90% of these embryos produce luciferase at the time when synthesis of zygotic gene products begins, regardless of the presence or absence of the F101 enhancer (Martínez-Salas *et al.*, 1989; Wiekowski *et al.*, 1991).

Each injected embryo received ~3×10^5 copies of plasmid DNA [~2 pl of 0.2 μg plasmid (6 kb)/μl buffer] and produced from 10^3 to 10^5 copies of luciferase mRNA. Luciferase mRNA was detected by isolating total RNA from a single injected embryo, and then amplifing a unique 336 nucleotide sequence within luciferase mRNA using RT–PCR technology. A fixed amount of luciferase-Δ36 RNA, which contained a 36 nucleotide deletion within the amplified sequence, was added to each sample of embryos before extraction to provide an internal standard. Both nascent luciferase mRNA and truncated luciferase-Δ36 RNA were purified and amplified simultaneously. The DNA products were then fractionated by gel electrophoresis and quantified using a PhosphorImager (Figure 2). Amplification of luciferase-Δ36 RNA was linear over a range of 10–10^5 copies. No signal was observed in embryos extracted immediately after plasmid injection, confirming that RNA preparations were not contaminated with copies of the injected luciferase gene.

Production of luciferase gene mRNA in S phase-arrested one-cell mouse embryos occurred in two phases. Transcription was first detected ~1.5 h after injection and increased thereafter (Figures 2 and 6A), demonstrating that S phase-arrested one-cell mouse embryos were transcriptionally competent. Since the ability of fertilized eggs to either transcribe or express the injected luciferase gene varied from one female to another, a biphasic time course was most evident when transcription was analyzed in fertilized eggs from a single pregnant female (Figure 3A). The greater the ability of one-cell embryos to transcribe the injected plasmid, the more clearly one could detect two phases of transcription. Since the amount of luciferase mRNA detected in single embryos followed a similar time course for each female, it was possible to shift individual

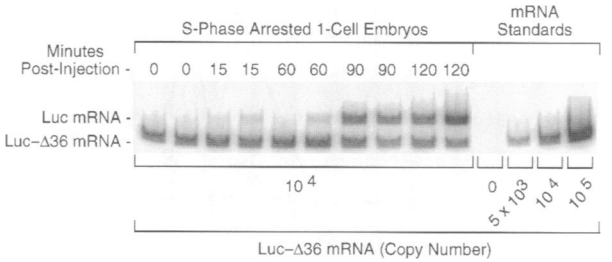

Fig. 2. Quantitative analysis of transcription from a plasmid-encoded gene injected into mouse embryos. Fertilized eggs isolated from a single female were cultured in the presence of aphidicolin to arrest cells as they entered S phase. At 24 h post-hCG, pPyluc was injected into their paternal pronucleus. Single embryos were harvested at the times indicated, and 10^4 copies of luciferase-Δ36 RNA (Luc-Δ36 RNA) were added to each embryo to provide an internal standard that would correct for variation in mRNA recovery and detection. Total RNA was then purified from each sample and a unique sequence present in both the nascent luciferase mRNA (Luc mRNA) and the internal standard RNA was amplified by RT–PCR in the presence of [α-^{32}P]dCTP. The DNA amplification products consisted of a 336 bp DNA fragment representing the luciferase mRNA and a 300 bp DNA fragment representing the internal standard RNA. These [^{32}P]DNA fragments were separated by gel electrophoresis and the amount of ^{32}P present in each band was quantified using a PhosphorImager. Standard curves consisting of different amounts of Luc-Δ36 RNA (example shown) were included routinely to assess the linearity of the RT–PCR reaction. The ratio of Luc mRNA to Luc-Δ36 RNA was used to calculate the relative amounts of luciferase mRNA present at various times after injection. Similar results were obtained with pF101Pyluc.

time courses (~3 h) to place their maxima in register (Figure 3B).

The mean values for five different females revealed a distinctive biphasic time course for accumulation of luciferase mRNA (Figure 3C). Transcription of the luciferase gene began upon injection of plasmid DNA at 25 h post-human chorionic gonadotropin (hCG), and luciferase mRNA accumulated during the late one-cell stage until it reached a peak at 40–44 h post-hCG. Nascent luciferase mRNA then fell to its lowest level at 45–47 h post-hCG before a second phase of luciferase mRNA accumulation occurred to produce a peak at 48–50 h post-hCG. This biphasic accumulation of luciferase mRNA over a 30 h time course most probably resulted from changes in the synthesis of luciferase mRNA rather than changes in its degradation, because luciferase mRNA is not particularly stable in cultured cells [$t_{1/2}$ = 6 h (Thompson *et al.*, 1991)], and mRNA is degraded rapidly in fertilized eggs. Specific mRNAs injected into the cytoplasm of fertilized mouse eggs are degraded with a half-life of 3–5 h (Ebert *et al.*, 1984), consistent with the fact that degradation of maternally inherited mRNA is triggered when the oocyte undergoes meiotic maturation and is ~90% completed by the two-cell stage in development (Schultz, 1993). Addition of α-amanitin to S phase-arrested one-cell embryos at the time they were injected reduced luciferase mRNA accumulation to 15% of control by 41 h post-hCG and 2% by 50 h, indicating a $t_{1/2}$ for nascent mRNA of <5 h.

While most of the one-cell embryos that were injected and cultured in the absence of aphidicolin developed into two-cell embryos, some of them spontaneously arrested development and remained morphologically as one-cell embryos with two pronuclei. The levels of luciferase mRNA and enzyme activity in these spontaneously

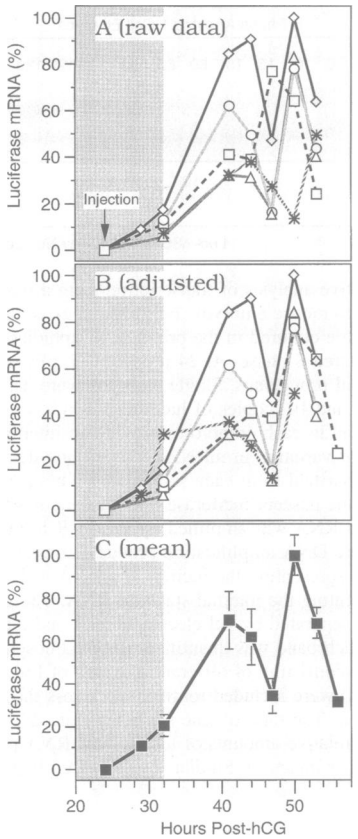

Fig. 3. Time course for transcription from an RNA polymerase II-dependent gene injected into S phase-arrested one-cell embryos. At 24-h post-hCG, pPyluc was injected into the paternal pronucleus of S phase-arrested one-cell embryos, and single embryos were harvested at the times indicated and then assayed for luciferase mRNA as described in Figure 2. The amount of luciferase mRNA was expressed relative to its peak value: the highest ratio of luciferase mRNA to luciferase-Δ36 RNA was defined as 100%. The maximum ratio depended on the amount of luciferase-Δ36 RNA that was added to the sample. In this experiment, the ratio at 50 h post-hCG was 3.5×10^6 c.p.m./1.5×10^5 c.p.m. = 23.3. Each data point represented the mean of five embryos. The standard error of the mean (omitted in A and B for the sake of clarity) was between 15 and 35% of the mean value, depending on the number of embryos analyzed. (**A**) Luciferase mRNA produced in S phase-arrested one-cell embryos from five different females. (**B**) Time courses shown in (A) after they were adjusted to place peaks in register. (**C**) Mean values for the time courses shown in (B). Error bars indicate standard error of the mean (± SEM). The shaded region indicates the duration of the average one-cell embryo before it cleaved into a two-cell embryo in experiments where aphidicolin was omitted. Similar results were obtained with pF101Pyluc.

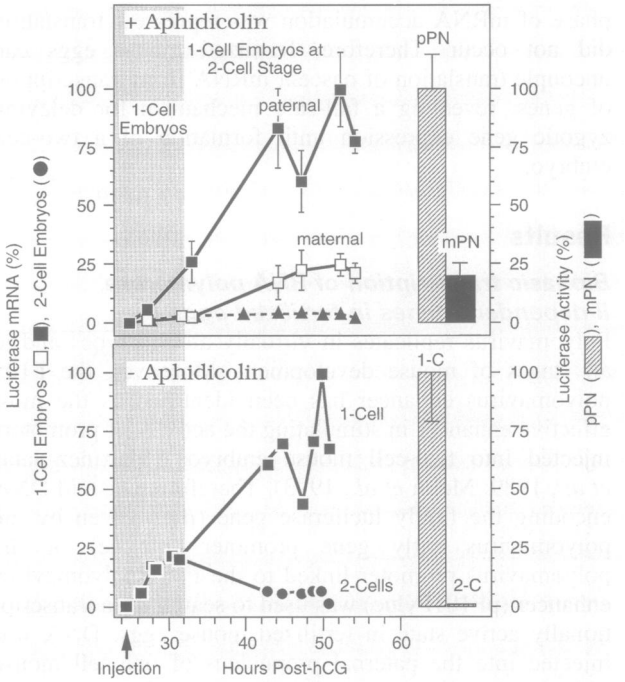

Fig. 4. (+ Aphidicolin) Transcriptional capacity of paternal and maternal pronuclei. At 24 h post-hCG, either pPyluc (■, □) (containing the luciferase gene driven by the polyomavirus promoter), or pluc (▲) (the same plasmid without the polyomavirus promoter) was injected into either the paternal pronucleus (pPN, ■, ▲) or maternal pronucleus (mPN, □) of S phase-arrested one-cell embryos as described in Figure 2. At various times after injection, S phase-arrested one-cell embryos were harvested and assayed individually for luciferase mRNA (■, □, ▲). Luciferase enzyme activity (pPN injections ▨, mPN injections ■) was assayed at 60 h post-hCG. Results from three independent experiments were combined. At 52 h post-hCG, the ratio of luc mRNA/luc-Δ36 was 7.2×10^6 c.p.m./2.3×10^5 c.p.m. = 31.3 in the paternal pronucleus and 3.1×10^6 c.p.m./5.8×10^5 c.p.m. = 5.3 in the maternal pronucleus. (– Aphidicolin) Effect of cell cleavage on the transcriptional capacity of one-cell embryos. The experiment described above was repeated in the absence of aphidicolin. More than 90% of these embryos underwent cleavage at ~34 h post-hCG to form two-cell embryos (●), although some arrested spontaneously and remained morphologically one-cell embryos with two pronuclei (■). The shaded region indicates the duration of the average one-cell embryo before it cleaved into a two-cell embryo in experiments where aphidicolin was omitted. At 50 h post-hCG, the mean ratio in spontaneously arrested one-cell embryos was 7.3×10^6 c.p.m./1.3×10^6 c.p.m. = 5.6. In two-cell embryos, the mean ratio was 1.9×10^5 c.p.m./1.7×10^6 c.p.m. = 0.1. Neither injection of pF101Pyluc into developing one-cell embryos nor addition of aphidicolin to some of the newly formed two-cell embryos stimulated luciferase gene transcription (data not shown).

arrested one-cell embryos were equivalent to those observed in aphidicolin-arrested one-cell embryos. Furthermore, the time course for luciferase mRNA synthesis in these spontaneously arrested one-cell embryos was strikingly similar to that in aphidicolin-treated, S phase-arrested one-cell embryos (compare Figure 4A and B). Therefore, biphasic transcription of genes injected into S phase-arrested one-cell embryos did not result from the presence of aphidicolin *per se*, but from the physiological state of the cell. Differences in the actual levels of luciferase mRNA produced in the presence or absence of aphidicolin reflected the place in the cell cycle where cells were arrested. For example, one-cell embryos arrested in G_2 phase by cytochalasin D did not express the luciferase gene (Martínez-Salas *et al.*, 1989).

Requirements for expression of injected genes

The requirements for transcription of the injected luciferase gene were consistent with the requirements for transcription of cellular genes. Both phases of luciferase gene transcription were dependent upon a functional promoter and pol II, since either deletion of the polyomavirus early gene promoter (pluc, Figure 4A) or addition of 10 μg/ml α-amanitin to the culture medium eliminated transcription (data not shown). As previously reported in studies measuring production of luciferase enyzme activity, production of luciferase mRNA in S phase-arrested one-cell embryos was not increased by linking the promoter to the F101 enhancer. Stimulation by the F101 enhancer was observed only when plasmid DNA was injected into two-cell embryos.

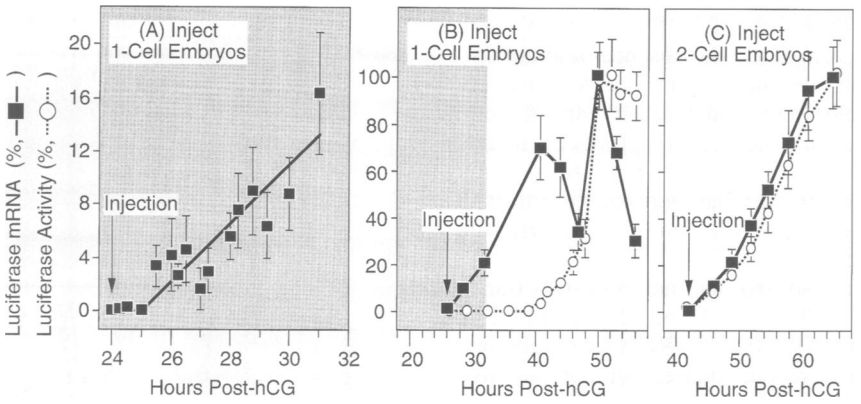

Fig. 5. Uncoupling of transcription and translation during zygotic gene activation in the mouse. Fertilized eggs were isolated at 18 h post-hCG. One group was cultured in the presence of aphidicolin to arrest them as they entered S phase, while a second group was cultured in the absence of aphidicolin in order to produce two-cell embryos. pF101Pyluc was injected into the paternal pronucleus of S phase-arrested one-cell embryos at 24 h post-hCG (**A** and **B**), and into one of the two nuclei of two-cell embryos at 42 h post-hCG (**C**). Luciferase mRNA (■) and luciferase enzyme activity (○) were then measured in individual embryos at the times indicated. Each data point represented at least five independent assays, and the error bars indicate ± SEM.

The same level of α-amanitin that prevented luciferase mRNA production also prevented expression of the 70 kDa 'transcription-requiring complex' (TRC; Poueymirou and Schultz, 1989), proteins that are among the first zygotic genes to be expressed (Figure 6C). Synthesis of these proteins is also sensitive to protein kinase A inhibitors (Poueymirou and Schultz, 1989). Consistent with this observation, 2.5 mM 6-dimethylaminopurine (a general inhibitor of protein kinase activity) inhibited luciferase gene transcription (data not shown). Furthermore, transcription of the *hsp70* gene [one of the first zygotic genes to be expressed (Bensaude *et al.*, 1983)] does not require prior protein synthesis (Manejwala *et al.*, 1991). Consistent with this observation, transcription of the injected luciferase gene was not inhibited by 10 μg/ml of cycloheximide, a concentration that completely blocked protein synthesis in one-cell mouse embryos (Figure 6C). Therefore, the same cellular factors are required for transcription of both injected genes and cellular genes, and the transcription machinery active in late one-cell and early two-cell embryos must be inherited from the oocyte.

Previous studies observed that injection of the luciferase gene into the paternal pronucleus of S phase one-cell embryos produced ~5-fold more luciferase enzyme activity than did injection of the same plasmid into the maternal pronucleus (Martínez-Salas *et al.*, 1989; Wiekowski *et al.*, 1993). To determine whether this difference resulted from differences in DNA transcription or mRNA translation, transcription of the luciferase gene was examined under the same conditions. These results revealed that the injected gene was transcribed ~4-fold more in the paternal pronucleus than in the maternal pronucleus (Figure 4A). Therefore, differences observed in the levels of luciferase enzyme activity following injection of plasmid DNA into mouse embryos reflected differences in the level of luciferase gene transcription. This is consistent with the short half-life of luciferase protein (Thompson *et al.*, 1991).

Translation is delayed until the two-cell stage in development

Previous studies have shown that the appearance of luciferase enzyme activity following injection of the luciferase gene into S phase-arrested one-cell mouse embryos was delayed until 44–48 h post-hCG (Martínez-Salas *et al.*, 1989; Wiekowski *et al.*, 1991), suggesting that synthesis of luciferase protein coincided with the second accumulation of luciferase mRNA. To test this hypothesis, pF101Pyluc was injected into S phase-arrested one-cell embryos and the time courses for the appearance of luciferase mRNA and luciferase enzyme activity were compared.

Luciferase mRNA appeared after a 1 h delay (Figure 5A) and then accumulated until ~40 h post-hCG (Figure 5B). However, significant levels of luciferase enzyme activity were not detected until after 40 h post-hCG, and its time course corresponded to that of the second phase of luciferase mRNA accumulation (Figure 5B). Thus, nascent mRNA produced during the late one-cell/early two-cell stage was not translated immediately. Translation of nascent mRNA was delayed until the late two-cell stage (G_2 phase) and coincided with the second phase of luciferase mRNA accumulation.

These results suggested that translation of nascent mRNA was not coupled with its transcription until after zygotic gene expression had begun. To test this hypothesis, pF101Pyluc was injected into late two-cell mouse embryos that were actively expressing their zygotic genes (described below). The results confirmed that by ~40 h post-hCG, translation of nascent luciferase mRNA was tightly coupled to transcription of the luciferase gene (Figure 5C). This experiment could not be carried out with S phase-arrested late one-cell embryos, because their ability to express either injected or endogenous genes declines rapidly after ~40 h post-hCG (Wiekowski *et al.*, 1991).

The data presented in Figure 5 suggested that translation of nascent mRNA in S phase-arrested one-cell embryos was tightly coupled to the second phase of mRNA accumulation that began ~40 h post-hCG. If this hypothesis were correct, then repression of the second phase of luciferase mRNA accumulation should prevent the appearance of luciferase enzyme activity. Previous studies revealed that expression of genes injected into one-cell embryos that continue development in the absence of aphidicolin is strongly repressed when these embryos undergo cleavage (Martínez-Salas *et al.*, 1989; Wiekowski *et al.*, 1993;

Henery *et al.*, 1995). Therefore, pF101Pyluc was injected into one-cell embryos cultured in the absence of aphidicolin. Under these conditions, most of the injected one-cell embryos underwent cleavage, and the amounts of both luciferase mRNA and enzyme activity produced in the resulting two-cell embryos were strongly repressed (Figure 4, –Aphidicolin), demonstrating that repression of injected genes during cleavage of one-cell embryos occurred at the level of transcription.

These data also demonstrated that luciferase was translated primarily, if not exclusively, from mRNA produced during the second phase of luciferase mRNA accumulation. Cleavage prevented the second phase, but not the first phase, of luciferase mRNA accumulation, and the first phase of mRNA accumulation in paternally injected embryos was equivalent in amount to the second phase of mRNA accumulation in maternally injected embryos. Since maternally injected embryos produced at least 20% as much luciferase enzyme as paternally injected embryos, one would expect a similar amount in paternally injected embryos that underwent cleavage. However, the amount of luciferase enyzme produced in these embryos was <1% of the amount produced in S phase-arrested one-cell embryos. Therefore, the nascent mRNA produced in late one-cell embryos contributed little to production of luciferase enzyme.

This conclusion was confirmed by the effects of α-amanitin, a specific inhibitor of pol II. Addition of α-amanitin to S phase-arrested one-cell embryos at 0, 4 or 8 h after injection of the luciferase gene prevented or blocked the appearance of luciferase enzyme activity (M.Wiekowski and M.Miranda, unpublished data), consistent with productive transcription of luciferase mRNA beginning at or slightly before translation of luciferase mRNA.

Expression of injected genes is linked to expression of zygotic genes

How is expression of injected genes related to expression of endogenous embryonic genes? Previous studies have identified two changes in the pattern of protein synthesis in cleavage stage embryos that are diagnostic for the onset of zygotic gene expression. The first is the TRC, whose synthesis is inhibited by α-amanitin, a specific inhibitor of pol II and pol III (Conover *et al.*, 1991). The second is a protein(s) of ~35 kDa that is translated from maternally inherited mRNA, but whose synthesis decreases when ZGA occurs. This decrease is inhibited by α-amanitin (Wiekowski *et al.*, 1991). A large number of other proteins are synthesized during this time period whose synthesis is not disturbed by α-amanitin. These nascent proteins are translated from maternally inherited mRNA. Numerous proteins are synthesized after 42 h post-hCG that represent the late phase of zygotic gene expression in two-cell embryos (Flach *et al.*, 1982; Bolton *et al.*, 1984; Latham *et al.*, 1991).

In order to compare the time course for luciferase gene expression with that for embryonic gene expression, nascent embryonic proteins were radiolabeled with [^{35}S]methionine as one-cell embryos developed into two-cell embryos either in the absence (Figure 6A) or presence (Figure 6C) of α-amanitin. TRC synthesis began ~36 h post-hCG, 3–4 h after the first mitosis (Figure 1). This was

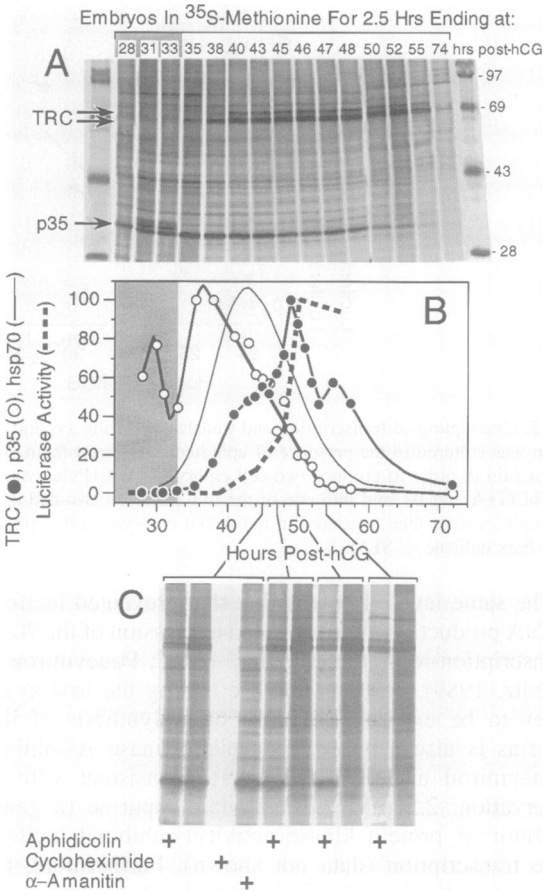

Fig. 6. Changes in cellular gene expression as one-cell mouse embryos develop into two-cell embryos. (**A**) Fertilized eggs were isolated at 18 h post-hCG and cultured with [^{35}S]methionine (Amersham, >1000 Ci/mmol) for 2.5 h periods ending at the indicated times post-hCG. Proteins were fractionated by electrophoresis in 10% SDS–polyacryamide gels as previously described (Wiekowski *et al.*, 1991), and the fraction of various ^{35}S-labeled proteins was quantified using a PhosphorImager. Total protein synthesis was determined from the entire gel lane. Molecular weight standards (Amersham) were fractionated in parallel lanes. (**B**) The total amount of radiolabel present in the 'transcription-requiring complex' (TRC) of ~70 kDa proteins and the 35 kDa protein(s) was determined for each lane in (A) and compared relative to the maximum value obtained in each experiment. Results from two experiments were combined. Mitosis occurred at 34 ± 2 h post-hCG. For comparison, expression of the injected luciferase gene (Figure 5) and an *hsp70*-driven luciferase transgene (Christians *et al.*, 1995) are also indicated. (**C**) Embryos (25 per lane) were incubated with 4 μg/ml aphidicolin, 10 μg/ml cycloheximide or 10 μg/ml of α-amanitin, and then radiolabeled for 2.5 h ending at the times indicated.

consistent with the published time course for expression of the TRC (Bolton *et al.*, 1984), and of *hsp70* (Figure 6B), one of the first zygotic genes expressed during mouse development (Christians *et al.*, 1995). A second, more pronounced phase of protein synthesis began ~44 h post-hCG, reaching its maximum rate of synthesis between 47 and 52 h post-hCG (Figure 6B). Repression of p35 synthesis began ~38 h post-hCG and was completed by 52 h post-hCG (Figure 6A and B). The slower migrating bands that appear with p35 from 31 to 33 h post-hCG indicate that p35 is phosphorylated during mitosis (Figure 6A and B). These data are consistent with previous descriptions of protein synthesis in fertilized mouse eggs

from inbred hybrid mice (Bolton *et al.*, 1984; Howlett and Bolton, 1985).

TRC expression and p35 repression followed the same time course in S phase-arrested one-cell embryos as in developing embryos, but total protein synthesis was significantly reduced (Figure 6C). Total protein synthesis began decreasing at ~35 h post-hCG (Wiekowski *et al.*, 1991). Comparison of zygotic gene expression with expression of pF101Pyluc injected 24 h post-hCG revealed that luciferase mRNA translation was concomitant with the late phase of zygotic gene translation in two-cell embryos (Figure 6B), confirming that expression of plasmid-encoded genes is regulated by the same mechanisms that regulate zygotic gene expression.

Biphasic transcription of RNA polymerase I- and III-dependent genes in fertilized eggs

Are fertilized mouse eggs competent for transcription of pol I and pol III-dependent promoters as well as for pol II-dependent promoters? To address this question, plasmids were injected into S phase-arrested one-cell embryos that encoded either the *Escherichia coli* chloramphenicol acetyl transferase (CAT) reporter gene under the control of the pol I-dependent ribosomal DNA promoter, enhancer and termination sequences (pE-MENAT, Palmer *et al.*, 1993), or the pol III-dependent adenovirus VA1 RNA gene (pMLVA1, Fowlkes and Shenk, 1980).

In order to compare these genes directly with the pol II-dependent polyomavirus promoter used in the experiments described above, the pol I- and pol III-dependent promoter constructions were each co-injected with an equal amount of pF101Pyluc or pPyluc. Luciferase-Δ36 RNA was added to each embryo before lysis to provide an internal standard to normalize luciferase gene transcription. Transcription of the co-injected pol I- or pol III-dependent gene was then normalized to luciferase gene transcription. This approach was taken because some of the *cis*-acting elements of the VA1 promoter are localized inside the VA1 RNA gene, making it difficult to link this promoter to a reporter gene. In addition, the CAT reporter gene was found to lack the sensitivity required to measure rRNA promoter activity.

The results demonstrated that S phase-arrested one-cell embryos could transcribe pol I-, pol II- and pol III-dependent promoters within 1–2 h of their injection (Figure 7). Moreover, transcription from the pol I- and pol III-dependent promoters followed the same biphasic time course observed for transcription from the pol II-dependent promoter, suggesting that activation of all three types of transcription in fertilized mouse eggs is governed by the same mechanism.

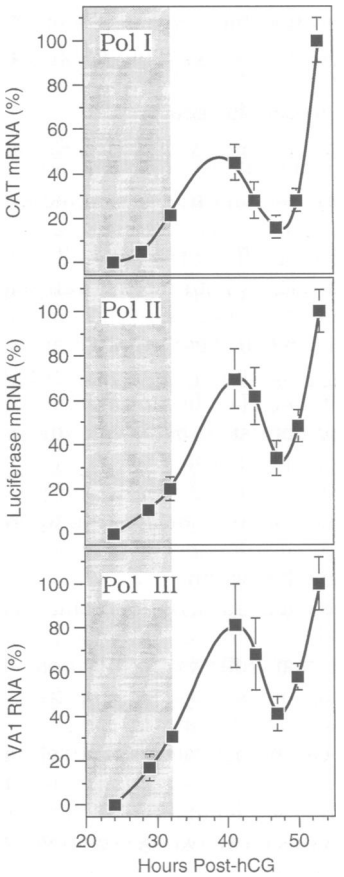

Fig. 7. Comparison of transcription activity from RNA polymerase I-, II- and III-dependent genes injected into S phase-arrested one-cell embryos. Embryos from a single female were injected with a DNA sample containing 0.1 μg/μl each of pE-MENAT + pF101Pyluc (Pol I) or pMLVAI + pF101Pyluc (Pol III). Luciferase-Δ36 RNA was added to the injected embryos prior to lysis in order to provide an internal standard by which to measure expression of the co-injected luciferase reporter gene (Pol II). The amount of luciferase mRNA produced at each time point was normalized to a constant amount of luciferase-Δ36 RNA. The same normalization factors were then used to adjust the amounts of VA1 RNA or CAT mRNA produced in the same embryos. Points were connected using an interpolation program. Each data point was the average of three independent assays.

Discussion

Transient expression of plasmid-encoded reporter genes reveals the embryo's capacity to carry out specific functions such as transcription or translation using specific *cis*-acting sequences and *trans*-acting factors. Studies presented here demonstrated that the capacity to transcribe genes is ~5-fold greater in paternal pronuclei than in maternal pronuclei, that transcription is repressed during the first cell cleavage and that enhancers are required to stimulate transcription in two-cell embryos, but not in the paternal pronucleus of S phase-arrested one-cell embryos. These results confirm, at the mRNA level, previous conclusions based on expression of reporter genes. Results presented here and in previous publications further show that expression of these genes is regulated by the same factors that regulate expression of embryo genes (Majumder and DePamphilis, 1995; Nothias *et al.*, 1995). Synthesis of plasmid gene proteins does not occur until zygotic gene proteins are synthesized. Plasmid gene transcription and expression both require specific promoter and enhancer sequences, occur only in cells that are competent for transcription and translation, such as growing oocytes and embryos at the two-cell stage or beyond, and are sensitive to inhibitors of transcription or translation. Finally, the same firefly luciferase gene construction used in the experiments described here has also been used to construct transgenic mice expressing luciferase driven by the mouse *hsp70* promoter, one of the first promoters to exhibit activity upon ZGA (Christians *et al.*, 1995). This luciferase transgene was expressed in a transient

α-amanitin-sensitive time course (Figure 6B) that mimicked activation of the endogenous *hsp70* promoter. Therefore, the results with plasmid-encoded genes should reflect what happens to cellular genes.

Results presented here and elsewhere reveal the surprising discovery that while late one-cell embryos are capable of both transcription and translation, these two processes are not coupled. Thus, the zygotic clock can delay transcription of genes until the late one-cell stage and translation of nascent mRNA until the two-cell stage. Moreover, mRNA synthesized in late one-cell embryos is translated poorly, if at all, when these embryos undergo cleavage, thus restricting the initial expression of zygotic genes entirely to the two-cell embryo. As discussed below, these conclusions are consistent both with studies on plasmid-encoded genes and with studies on zygotic gene activation. Thus, fertilized eggs in the mouse, and presumably other mammals, contain a novel mechanism to prevent promiscuous transcription from adversely affecting activation of the developmental program.

Such unscheduled transcription could occur as a consequence of the extensive chromatin remodeling and reorganization that follows fertilization. Both sets of parental chromosomes must be converted from a condensed meiotic state to one in which most genes are repressed but can be activated selectively as appropriate promoter and enhancer transcription factors become available. In the mouse, repression is apparent during the conversion of one-cell to two-cell embryos when the two parental genomes are reorganized into a single zygotic nucleus. Remodeling is particularly dramatic in the paternal genome, because it passes from a state in which DNA is packaged exclusively with protamines, through a state in which neither protamines nor histones are detectable, to one in which it is packaged exclusively with histones (Rodman *et al.*, 1981; Adenot *et al.*, 1991). Thus, it would be developmentally advantageous if expression of zygotic genes was delayed until this remodeling and reorganization was completed.

Uncoupling translation from transcription in plasmid-encoded genes

The results presented here demonstrate clearly that late one-cell mouse embryos are competent for transcription of pol I-, II- or III-dependent genes, but they cannot translate nascent mRNA efficiently. Fertilized mouse eggs can, however, translate efficiently pre-formed mRNA that is injected into their cytoplasm as early as 20 h post-hCG [α-globin mRNA (Ebert and Brinster, 1983), *E.coli* lacZ mRNA (Vernet *et al.*, 1992), GAL4:VP16 mRNA (S.Majumder, unpublished data)]. Therefore, one-cell embryos can distinguish nascent mRNA from pre-formed (i.e. maternally inherited) mRNA. An analogous result has been observed in *Xenopus* where natural mRNA synthesized within the oocyte nucleus is translated with an efficiency ~50 times less than when the same mRNA is injected into the oocyte cytoplasm (Bouvet and Wolffe, 1994).

Translation of nascent mRNA is delayed until zygotic genes are expressed at the two-cell stage in development. Moreover, translation occurs predominantly, if not exclusively, from transcripts produced ≥40 h post-hCG (early two-cell stage). Expression of pol II-dependent luciferase activity following injection of plasmid DNA into the paternal pronucleus of S phase-arrested one-cell embryos was not detected until ~40 h post-hCG (Figure 5B), consistent with previous studies (Martínez-Salas *et al.*, 1989; Wiekowski *et al.*, 1991). This corresponded to the late phase of ZGA following the initial synthesis of transcription requiring proteins such as *hsp70* (Figure 6B). Surprisingly, this delay was as much as 15 h after luciferase mRNA first appeared (Figure 5A). In contrast, the appearance of luciferase enzyme activity and mRNA were coincident following plasmid injection into two-cell embryos that were actively expressing their zygotic genes (Figure 5C). Therefore, while late one-cell embryos were capable of transcription, nascent transcripts were not translated until the two-cell stage in development.

Accumulation of pol II-dependent luciferase mRNA (Figure 3) as well as pol I- and pol III-dependent transcripts (Figure 7) was biphasic, with transcription beginning almost immediately after DNA was injected into late one-cell embryos (Figure 5A). This biphasic time course most probably resulted from two bursts of luciferase mRNA synthesis, one immediately after injection and the second when zygotic genes underwent transcription, because both injected and maternally inherited mRNA is degraded rapidly in fertilized eggs with a half-life of 3–5 h (Ebert *et al.*, 1984; Schultz, 1993). Since the same biphasic time course was observed in both spontaneously arrested and aphidicolin-arrested one-cell embryos (Figure 4), the presence of this drug did not alter the mRNA synthesis/degradation profile. Biphasic transcription also has been observed when plasmid-encoded pol II- and pol III-dependent genes were introduced into *Xenopus* eggs or egg extracts (Newport and Kirschner, 1982; Prioleau *et al.*, 1994; Almouzni and Wolffe, 1995). The initial phase of transcription was repressed as the injected plasmid DNA was assembled into chromatin, and the time required for repression was related to the efficiency of chromatin assembly. Transcription of this chromatin template then resumed with the onset of zygotic gene expression at the mid-blastula transition. Thus, the biphasic accumulation of nascent RNA most likely reflects the conversion of bare DNA into a mini-chromosome. The initial DNA template is accessible to transcription factors present at the late one-cell stage, but the subsequent chromatin that forms (Martínez-Salas *et al.*, 1989) is refractory to transcription until ZGA. Chromatin-mediated repression occurs when one-cell embryos cleave into two-cell embryos (Henery *et al.*, 1995; Majumder and DePamphilis, 1995; Nothias *et al.*, 1995), and this repression acts at the level of transcription (Figure 4B). The biphasic transcription time courses observed when bare DNA is injected into embryo nuclei (Figure 7) can thus be understood as competition between transcription factors present as early as the one-cell stage and chromatin-mediated repression that appears during the one- to two-cell transition.

Three lines of evidence support the conclusion that translation is linked only to nascent mRNA synthesized during the onset of zygotic gene expression at the two-cell stage in development. First, the appearance of luciferase enzyme activity began ~40 h post-hCG, concomitant with the second phase of luciferase mRNA accumulation (Figure 5B) which was concurrent with zygotic gene expression (Figure 6). Since translation of luciferase

mRNA was tightly linked to its synthesis when DNA was injected into two-cell embryos expressing their zygotic genes (Figure 5C), translation should be coupled to transcription in arrested one-cell embryos when ZGA occurs. Second, when one-cell embryos developed into two-cell embryos in the absence of aphidicolin (Figure 4, −Aphidicolin), nascent luciferase mRNA accumulated during the late one-cell stage, but not during the two-cell stage, and the amount of luciferase enzyme produced was negligible. Comparison of these results with those in which the maternal pronuclei had been injected revealed that significant amounts of luciferase enzyme should have been observed in unarrested embryos if the initial phase of transcription had been coupled to translation. Third, α-amanitin prevented production of luciferase enzyme when applied before ~40 h post-hCG (the beginning of the second luciferase mRNA accumulation). Thus, it appears that nascent mRNA produced in late one-cell embryos is translated poorly, if at all. Translation is coupled to mRNA synthesized during the onset of zygotic gene expression at the two-cell stage.

In contrast to the results reported here, two previous studies reported that expression of plasmid-encoded reporter genes could be detected in late one-cell mouse embryos (Vernet *et al.*, 1992; Ram and Schultz, 1993). In one study (Vernet *et al.*, 1992), 20–50% of the injected embryos were β-galactosidase positive prior to cleavage, although the amount of β-galactosidase activity produced per embryo was not assayed. Therefore, the relative efficiency of translation of nascent mRNA in one- and two-cell embryos was not determined. However, it was clear in this study that one-cell embryos were much more efficient at translating injected mRNA than nascent mRNA. In the other study (Ram and Schultz, 1993), late one-cell embryos produced ~20% as much firefly luciferase as did injected two-cell embryos in a 4 h period. In our experiments, the amount of luciferase produced by injected two-cell embryos in 4 h would constitute ~20% of the final amount of luciferase produced (Figure 5C). Therefore, the levels of luciferase observed by Ram and Schultz (1993) in late one-cell embryos may represent ~4% of the total output of an injected two-cell embryo. The extent to which the nascent mRNA is translated in one-cell embryos may depend on experimental conditions such as the amount of DNA injected, injection conditions, culture conditions and the fraction of injected one-cell embryos that spontaneously arrest development and subsequently undergo ZGA.

Uncoupling translation from transcription in cellular genes

The results discussed above from injection of plasmid DNA into mouse embryos demonstrate that the ability to transcribe genes is present in late one-cell embryos, but the ability to translate nascent mRNA does not appear until the two-cell stage. Do these conclusions also apply to cellular genes as well? The bulk of both transcription and translation of zygotic genes does not occur until the two-cell stage in mouse development. Nevertheless, transplantation of nuclei from two-cell stage embryos back into one-cell embryos has revealed that late one-cell embryos can support zygotic gene expression once ZGA has been initiated (Latham *et al.*, 1992). In fact, transcription has been detected in late one-cell mouse embryos by incorporation of labeled nucleotides (Clegg and Piko, 1983; Bouniol *et al.*, 1995), increased intensity of hybridization to RNAs containing B2 repeats (Vasseur *et al.*, 1985), changes in relative levels of specific mRNAs (Temeles *et al.*, 1994) and detection of specific transgene mRNAs (Matsumoto *et al.*, 1994; Christians *et al.*, 1995). However, the proteins encoded by these mRNAs are not detected until the two-cell stage, suggesting that early mRNAs may be translated inefficiently.

Transgenic mice expressing luciferase driven by the mouse *hsp70* promoter, one of the first promoters to exhibit activity upon ZGA, produced luciferase enzyme activity in a transient α-amanitin-sensitive time course (Figure 6B) that mimicked activation of the endogenous *hsp70* promoter (Christians *et al.*, 1995). This transgene was introduced by the sperm into the eggs of non-transgenic females. About 12% of the resulting fertilized eggs produced luciferase enzyme activity and the level of activity was only ~2% of that observed in two-cell embryos. A similar result was obtained using sperm carrying a firefly luciferase gene driven by the β-actin promoter (Matsumoto *et al.*, 1994). Luciferase mRNA was detected in late one-cell embryos, but luciferase enzyme activity was not detected until ~10 h later, concomitant with formation of two-cell embryos. Thus, while late one-cell embryos are capable of initiating transcription on bare plasmid DNA, they are inefficient at initiating transcription of their endogenous chromosomes, and the transcripts that are produced are translated poorly, if at all. Thus, the bulk of zygotic gene expression from the paternal pronucleus is delayed until formation of a two-cell embryo, consistent with the results of plasmid gene expression described here.

Mechanisms for delaying transcription and translation

The zygotic clock regulates expression from plasmid-encoded reporter genes injected into the nuclei of fertilized eggs as well as cellular genes. Therefore, the ability to delay gene expression at the beginning of mammalian development does not depend on a unique post-meiotic chromatin structure associated with the genomes of gametes, but instead involves *trans*-acting factors that prevent transcription and/or translation of genes. The facts that transcription from pol I-, II- and III-dependent promoters injected into late one-cell embryos exhibits similar time courses (Figure 7) and that the bulk of transcription from all three classes of promoters is delayed until the late one-cell/two-cell stage in mouse development (see Introduction) suggests that the zygotic clock delays all transcription through a common transcription factor. A likely candidate is the TATA-box binding protein (TBP), a factor crucial for assembling active transcription complexes with all three RNA polymerases (White and Jackson, 1992). TBP is present in mouse oocytes and one-cell embryos (Worrad *et al.*, 1994), but regulation of its phosphorylated state could provide a mechanism to delay the ability of embryos to form active transcription complexes until the late one-cell stage in development.

Even when pol II transcription does occur in late one-cell embryos, the nascent mRNA is not translated. Three mechanisms could account for this. The first involves mRNA stability. While mouse oocytes and one-cell embryos (early as well as late) are capable of translating mRNAs injected into their cytoplasm (Ebert and Brinster, 1983; Ebert *et al.*, 1984; Vernet *et al.*, 1992), mRNA injected into

one-cell embryos is degraded ~65-fold faster than mRNA injected into oocytes (Ebert *et al.*, 1984). This could explain why nascent cellular RNA could be detected in the nucleus but not in the cytoplasm of one-cell mouse embryos (Bouniol *et al.*, 1995). The second involves mRNA processing. Regulating the length of the 3′ poly(A) tail on mRNAs controls translation of maternally inherited mRNAs in both frogs and mice (Paynton and Bachvarova, 1994; Wormington, 1994). Thus, if poly(A) tails in the initial phase of plasmid-encoded gene transcription are too short, these mRNAs may not be translated. The third involves mRNA translation. While *Xenopus* eggs can translate injected mRNAs, translation of nascent mRNAs can be suppressed by binding to specific proteins (Bouvet and Wolffe, 1994). Whatever the mechanism that prevents translation of newly synthesized mRNA in mouse one-cell embryos, it must be reversed following the first mitosis.

Materials and methods

Injection of DNA into mouse pre-implantation embryos

Experimental protocols and culture conditions have been described in detail (DePamphilis *et al.*, 1988; Miranda and DePamphilis, 1993). Plasmids have been described by Martínez-Salas *et al.* (1989). DNA (0.2 μg/μl) was injected into one of the pronuclei of one-cell embryos at 24 h post-hCG or into one of the zygotic nuclei of a two-cell embryo at 42 h post-hCG, and individual surviving embryos were assayed quantitatively for either luciferase enzyme activity as described by Miranda *et al.* (1993) or luciferase mRNA as described below.

Preparation of luciferase RNA internal standard

Quantitative analysis of mRNA produced from plasmids injected into mouse embryos required addition of an internal standard comparable with the cell extracts. The internal standard was constructed by cloning the 618 bp *Eco*RI–*Pst*I fragment of the firefly luciferase gene (isolated from the F101Pyluc) into the *Eco*RI and *Pst*I sites of pGEM-4Z (Promega). The structure of the firefly luciferase gene in these expression vectors is described by DeWet *et al.* (1987). This plasmid (pGEM-luc) was then digested with the *Bsi*WI restriction enzyme (NEB-Biolabs), and nested deletions from the resulting 5′-extended DNA fragments were performed by the Exo-Size deletion kit (NEB-Biolabs). One of these deletions was introduced into pGEM-4Z to produce pGEM-lucΔ, identical to pGEM-luc except that it contained a 36 bp deletion within the luciferase gene. The sequence around this deletion is ATTTCGAAGT-Δ-CTGTAAAAGC. Internal standard luciferase-Δ36 RNA (613 bp) was produced by transcribing the *Eco*RI-digested pGEM-lucΔ *in vitro* using the T7 promoter encoded by pGEM according to the manufacturer's instructions (Megascript kit, Ambion). RNA was purified by centrifugation through an RNase-free G40 column (Clontech). RNA dilutions were made fresh for each experiment from an RNA stock (200 μg/ml in RNase-free water) stored at –20°C. RNA concentration was determined by spectrophotometry.

Quantitative analysis of gene transcription in injected embryos

All solutions were RNase-free (Promega or Sigma RNase-free water). At appropriate times, each embryo was washed rapidly in phosphate-buffered saline (PBS), harvested in 10 μl of water, placed in its own 1.5 ml Eppendorf centrifuge tube and then frozen at –70°C. Samples were usually stored at –70°C for 1 week to 2 months. Polyinosinic acid (Pharmacia) (4 μg/embryo) was added as a carrier (Winslow and Henkart, 1991) to facilitate RNA recovery, and luciferase-Δ36 RNA (~10^4 copies/embryo) was added as an internal standard in order to correct for RNA losses during the subsequent extraction. Total RNA was then extracted by adding 200 μl of TRIzol according the manufacturer's protocol (GIBCO-BRL; Chomczynski, 1993). Injected plasmid DNA that might contaminate the extract was removed by resuspending the RNA in 100 μl of 40 mM Tris–HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$, and 1 U RNase-free DNase I (Promega) for 45 min at 37°C. The reaction was stopped by adding 10 μl of 5 M ammonium acetate, 100 mM EDTA before extracting once with an equal volume of phenol–chloroform–isoamylalcohol (pH 7.9; Amresco). RNA was precipitated from the aqueous phase by addition of 2.5 volumes of 100% ethanol, stored overnight at 4°C, recovered by centrifugation, washed by 75% EtOH, air dried, and resuspended in RNase-free water.

Luciferase mRNA and luciferase-Δ36 RNA present in embryo extracts were detected simultaneously using a reverse transcriptase–DNA polymerase chain reaction (RT–PCR) to copy and amplify both RNAs with the same primers. The amplicon encompassed the 36 nucleotide deletion in the internal standard RNA. To prevent contamination, aerosol-free pipet tips were used only once. Concentrated reaction solutions were prepared in batch and aliquoted to each tube to minimize pipeting errors after pellets were resuspended in water. The RNA sample was denatured at 70°C for 10 min and then reverse transcription was carried out in a total volume of 40 μl using 1 μg of antisense primer (GGTAGGCTGCG-AAATGTTCATACTG) and 100 U of Superscript II enzyme (GIBCO-BRL) for 2 h at 42°C in the 1× PCR buffer (Perkin Elmer RNA–PCR kit). The diluted enzyme was added in reaction medium pre-warmed to 42°C. DNA amplification was performed in Gene Amp thin walled reaction tubes (Perkin Elmer) using a 9600 thermocycler (Perkin Elmer) on 10 μl of the RT-DNA by adding 1 μg of each sense (GCCATTCTAT-CCTCTAGAGGATGGA) and antisense primer in the presence of 5 mCi of [α-^{32}P]dCTP, according to the Perkin Elmer RNA–PCR kit specifications. Conditions were 2 min at 95°C then 28 cycles at 15 s at 95°C, 30 s at 60°C and a final elongation for 7 min at 72°C. The resulting DNA products consisted of a 336 bp fragment from luciferase mRNA and a 300 bp fragment from luciferase-Δ36 RNA that were separated by electrophoresis in 6% acrylamide–agarose (AMRESCO) 20 cm gels in Tris–borate–EDTA buffer (CBS Scientific, 200 V). The acrylamide gel was transferred onto Whatman's 3MM paper by drying with a vacuum pump (Savant) and then exposed to phosphor screens (Kodak) using a 445 SI PhosphorImager (Molecular Dynamics) or to film for 1–12 h.

Variability in luciferase mRNA levels was markedly reduced by comparing embryos that were isolated and injected with the same needle on the same day in order to minimize differences in culture conditions, amount of DNA injected per embryo and cell cycle variability. Aliquots of freshly diluted stock solutions of luciferase-Δ36 RNA, primers and enzymes were added to each embryo extract.

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References

Adenot,P.G., Szöllösi,M.S., Geze,M., Renard,J.P. and Debey,P. (1991) Dynamics of paternal chromatin changes in live one-cell mouse embryo after natural fertilization. *Mol. Reprod. Dev.*, **28**, 23–24.

Almouzni,G. and Wolffe,A.P. (1995) Constraints on transcriptional activator function contribute to transcriptional quiescence during early *Xenopus* embryogenesis. *EMBO J.*, **8**, 1752–1765.

Bensaude,O., Babinet,C., Morange,M. and Jacob,F. (1983) Heat shock proteins, first major products of zygotic gene activity in mouse embryo. *Nature*, **305**, 331–333.

Bolton,V.N., Oades,P. and Johnson,M.H. (1984) The relationship between cleavage, DNA replication and gene expression in the mouse two-cell embryo. *J. Embryol Exp. Morphol.*, **79**, 139–163.

Bouniol,C., Nguyen,E. and Debey,P. (1995) Endogenous transcription occurs at the one-cell stage in the mouse embryo. *Exp. Cell Res.*, **218**, 57–62.

Bouvet,P. and Wolffe,A.P. (1994) A role for transcription and FRGY2 in masking maternal mRNA within *Xenopus* oocytes. *Cell*, **77**, 931–941.

Chomczynski,P. (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques*, **15**, 532–536.

Christians,E., Campion,E., Thompson,E.M. and Renard,J.P. (1995) Expression of the HSP 70.1 gene, a landmark of early zygotic activity in the mouse embryo, is restricted to the first phase of transcription. *Development*, **121**, 113–122.

Clegg,K.B. and Piko,L. (1983) Poly(A) length, cytoplasmic adenylation and synthesis of poly(A)$^+$ RNA in early mouse embryos. *Dev. Biol.*, **95**, 331–341.

Conover,J.C., Temeles,G.L., Zimmermann,J.W., Burke,B. and Schultz, R.M. (1991) Stage-specific expression of a family of proteins that are

major products of zygotic gene activation in the mouse embryo. *Dev. Biol.*, **144**, 392–404.

Dean,W.L., Seufert,A.C., Schultz,G.A., Prather,R.S., Simerly,C., Schatten,G., Pilch,D.R. and Marzluff,W.F. (1989) The small nuclear RNAs for pre-mRNA splicing are coordinately regulated during oocyte maturation and early embryogenesis in the mouse. *Development*, **106**, 325–334.

DePamphilis,M.L., Herman,S.A., Martínez-Salas,E., Chalifour,L.E., Wirak,D.O., Cupo,D.Y. and Miranda,M. (1988) Microinjecting DNA into mouse ova to study DNA replication and gene expression and to produce transgenic animals. *Biotechniques*, **6**, 662–680.

DeWet,J.R., Wood,K.V., DeLuca,M., Helinski,D.R. and Subramani,S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.*, **7**, 725–737.

Ebert,K.M. and Brinster,R.L. (1983) Rabbit α-globin mRNA translation by the mouse ovum. *J. Embryol. Exp. Morphol.*, **74**, 159–168.

Ebert,K.M., Paynton,B.V., McKnight,G.S. and Brinster,R.L. (1984) Translation and stability of ovalbumin mRNA injected into growing oocytes and fertilized ova of mice. *J. Embryol. Exp. Morphol.*, **84**, 91–103.

Flach,G., Johnson,M.H., Braude,P.R. and Bolton,V.N. (1982) The transition from maternal to embryonic control in the two-cell mouse embryo. *EMBO J.*, **1**, 681–686.

Fowlkes,D.M. and Shenk,T. (1980) Transcriptional control regions of the adenovirus VAI RNA gene. *Cell*, **22**, 405–413.

Geuskens,M. and Alexandre,H. (1984) Ultrastructural and autoradiographic studies of nucleolar development and rDNA transcription in preimplantation mouse embryos. *Cell Differ.*, **14**, 125–134.

Henery,C.C., Miranda,M., Wiekowski,M., Wilmut,I. and DePamphilis, M.L. (1995) Repression of gene expression at the beginning of mouse development. *Dev. Biol.*, **169**, 448–460.

Howlett,S. (1986) The effect of inhibiting DNA replication in the one-cell mouse embryo. *Wilhelm Roux's Arch. Dev. Biol.*, **195**, 499–505.

Howlett,S. and Bolton,V.N. (1985) Sequence and regulation of morphological and molecular events during the first cell cycle of mouse embryogenesis. *J. Embryol. Exp. Morphol.*, **87**, 175–206.

Kinloch,R.A. and Wassarman,P.M. (1993) Specific gene expression during oogenesis in mice. In Harford,J.B. (ed.), *Genes in Mammalian Reproduction*. Wiley-Liss, Inc., pp. 27–43.

Kopecny,V., Fléchon,J.E, Camous,S. and Fulka,J. (1989) Nucleogenesis and the onset of transcription in the eight-cell bovine embryo: fine-structural autoradiographic study. *Mol. Reprod. Dev.*, **1**, 79–90.

Latham,K.E., Garrels,J.I., Chang,C. and Solter,D. (1991) Quantitative analysis of protein synthesis in mouse embryos. I. Extensive reprogramming at the one- and the two-cell stages. *Development*, **112**, 921–932.

Latham,K.E., Solter,D. and Schultz,R.M. (1992) Acquisition of a transcriptionally permissive state during the one-cell stage of mouse embryogenesis. *Dev. Biol.*, **149**, 457–462.

Majumder,S. and DePamphilis,M.L. (1995) A unique role for enhancers is revealed during early mouse development. *BioEssays*, **17**, 879–889.

Manejwala,F.M., Logan,C.Y. and Schultz,R.M. (1991) Regulation of hsp70 mRNA levels during oocyte maturation and zygotic gene activation in the mouse. *Dev. Biol.*, **144**, 301–308.

Martínez-Salas,E., Linney,E., Hassell,J. and DePamphilis,M.L. (1989) The need for enhancers in gene expression first appears during mouse development with formation of the zygotic nucleus. *Genes Dev.*, **3**, 1493–1506.

Matsumoto,K., Anzai,M., Nakagata,N., Takahashi,A., Takahashi,Y. and Miyata,K. (1994) Onset of paternal gene activation in early mouse embryos fertilized with transgenic mouse sperm. *Mol. Reprod. Dev.*, **39**, 136–140.

Mélin,F., Miranda,M., Montreau,N., DePamphilis,M.L. and Blangy,D. (1993) Transcription enhancer factor-1 (TEF-1) DNA binding sites can specifically enhance gene expression at the beginning of mouse development. *EMBO J.*, **12**, 4657–4666.

Miranda,M. and DePamphilis,M.L. (1993) Preparation of injection pipettes. *Methods Enzymol.*, **225**, 407–411.

Miranda,M., Majumder,S., Wiekowski,M. and DePamphilis,M.L. (1993) Application of the firefly luciferase to preimplantation development. *Methods Enzymol.*, **225**, 412–433.

Newport,J. and Kirschner,M. (1982) A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell*, **30**, 687–696.

Nothias,J.-Y., Majumder,S., Kaneko,K.J. and DePamphilis,M.L. (1995) Regulation of gene expression at the beginning of mammalian development. *J. Biol. Chem.*, **270**, 22077–22080.

Palmer,T.D., Miller,A.D., Reeder,R.H. and McStay,B. (1993) Efficient expression of a protein coding gene under the control of an RNA polymerase I promoter. *Nucleic Acids Res.*, **21**, 3451–3457.

Paynton,B.V. and Bachvarova,R. (1994) Polyadenylation and deadenylation of maternal mRNAs during oocyte growth and maturation in the mouse. *Mol. Reprod. Dev.*, **37**, 172–180.

Paynton,B.V., Rempel,R. and Bachvarova,R. (1988) Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. *Dev. Biol.*, **129**, 304–314.

Piko,L. and Clegg,K.B. (1982) Quantitative changes in total RNA, total poly(A) and ribosomes in early mouse embryos. *Dev. Biol.*, **89**, 363–378.

Poueymirou,W.T. and Schultz,R.M. (1989) Regulation of mouse development: inhibition of synthesis of proteins in the two-cell embryo that require transcription by inhibitors of cAMP-dependent protein-kinase. *Dev. Biol.*, **133**, 588–599.

Prioleau,M.-N., Huet,J., Sentenac,A. and Méchali,M. (1994) Competition between chromatin and transcription complex assembly regulates gene expression during early development. *Cell*, **77**, 439–449.

Ram,P. and Schultz,R.M. (1993) Reporter gene expression in G2 of the one-cell mouse embryo. *Dev. Biol.*, **156**, 552–556.

Rodman,T.C., Pruslin,F.H., Hoffmann,H.P. and Allfrey,V.G. (1981) Turnover of basic chromosomal proteins in fertilized eggs: a cytoimmunochemical study of events *in vivo*. *J. Cell Biol.*, **90**, 351–361.

Schultz,G.A. and Heyner,S. (1992) Gene expression in pre-implantation mammalian embryos. *Mutat. Res.*, **296**, 17–31.

Schultz,R.M. (1993) Regulation of zygotic gene activation in the mouse. *BioEssays*, **15**, 531–538.

Takeuchi,I.K. and Takeuchi,Y.K. (1986) Ultrastructural localization of Ag-NOR proteins in full grown oocytes and preimplantation embryos of mice. *J. Electron Microsc.*, **35**, 280–287.

Taylor,K.D. and Piko,L. (1992) Expression of ribosomal protein genes in the mouse oocytes and early embryos. *Mol. Reprod. Dev.*, **31**, 182–188.

Temeles,G.L., Ram,P.T., Rothstein,J.L. and Schultz,R.M. (1994) Expression patterns of novel genes during mouse preimplantation embryogenesis. *Mol. Reprod. Dev.*, **37**, 121–129.

Thompson,J.F., Hayes,L.S. and Lloyd,D.B. (1991) Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene*, **103**, 171–177.

Vasseur,M., Condamine,H. and Duprey,P. (1985) RNAs containing B2 repeated sequences are transcribed in the early stages of mouse embryogenesis. *EMBO J.*, **4**, 1749–1753.

Vautier,D., Besombes,D., Chassoux,D., Aubry,F. and Debey,P. (1994) Redistribution of nuclear antigens linked to cell proliferation and RNA processing in mouse oocytes and early embryos. *Mol. Reprod. Dev.*, **38**, 119–130.

Vernet,M., Bonnerot,C., Briand,P. and Nicolas,J.F. (1992) Changes in permissiveness for the expression of microinjected DNA during the first cleavages of mouse embryos. *Mech. Dev.*, **36**, 129–139.

White,R.J. and Jackson,S.P. (1992) The TATA-binding protein: a central role in transcription by RNA polymerases I, II and III. *Trends Genet.*, **8**, 284–288.

Wiekowski,M., Miranda,M. and DePamphilis,M.L. (1991) Regulation of gene expression in preimplantation mouse embryos: effects of the zygotic clock and the first mitosis on promoter and enhancer activities. *Dev. Biol.*, **147**, 403–414.

Wiekowski,M., Miranda,M. and DePamphilis,M.L. (1993) Requirements for promoter activity in mouse oocytes and embryos distinguish paternal pronuclei from maternal and zygotic nuclei. *Dev. Biol.*, **159**, 366–378.

Winslow,S.G. and Henkart,P.A. (1991) Polyinosinic acid as a carrier in the microscale purification of total RNA. *Nucleic Acids Res.*, **19**, 3251–3253.

Wormington,M. (1994) Unmasking the role of the 3′-UTR in the cytoplasmic polyadenylation and translational regulation of maternal mRNAs. *Bioessays*, **16**, 533–535.

Worrad,D.M., Ram,P.T. and Schultz,R.M. (1994) Regulation of gene expression in the mouse oocyte and early preimplantation embryo: developmental changes in Sp1 and TATA box binding protein, TBP. *Development*, **120**, 2347–2357.

Yasuda,G.K. and Schubiger,G. (1992) Temporal regulation in the early embryo: is MBT too good to be true. *Trends Genet.*, **8**, 124–127.

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