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# Requirements for DNA Transcription and Replication at the Beginning of Mouse Development

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**Abstract** In mice, the first round of DNA replication occurs in fertilized eggs (1-cell embryos), while the onset of zygotic gene transcription begins ~20 hours after fertilization, a time that normally coincides with formation of a 2-cell embryo. One approach to investigating the mechanisms that control these developmentally regulated events has been to microinject plasmid DNA into the nuclei of mouse oocytes and embryos in order to determine the requirements for unique DNA sequences that regulate transcription and replication. The results from these and other studies have revealed two important mechanisms that regulate the beginning of animal development. The first is a time dependent "zygotic clock" of unknown detail that delays the onset of transcription, regardless of whether or not a 2-cell embryo is formed. The second is a mechanism that represses the activity of promoters and origins of replication specifically in maternal pronuclei of oocytes and 1-cell embryos, and in all nuclei of 2-cell embryos, regardless of their parental origin or ploidy. This repression is linked to chromatin, but the striking ability to relieve this repression with specific embryo-responsive enhancers first appears with formation of a 2-cell embryo. The need for a TATA-box to mediate enhancer stimulation of promoter activity appears even later when cell differention becomes evident. Thus, a biological clock delays transcription until both paternal and maternal genomes are replicated and remodeled from a post-meiotic state to one in which transcription is repressed by chromatin structure in a manner that can be relieved by cell-specific enhancers at appropriate times during development. 

**Key words:** DNA, transcription, replication, enhancer, TATA box, "zygotic clock"

In multicellular animals, transcription by RNA polymerase II is dependent primarily on three regulatory components: promoters, enhancers and locus control regions. Promoters, located proximal to the 5'-end of each gene, determine where transcription begins. Enhancers, located distal to either end of a gene, stimulate promoters in a tissue specific manner, independent of their orientation. Locus control regions consist of a collection of enhancer elements that operate at greater distances than traditional enhancers to provide a promoter independence from its chromosomal position [Grosveld et al., 1993]. Initiation of DNA replication bears similarities to initiation of transcription. The origin of replication consists of a core component that is analogous to a transcription promoter; it determines where DNA replication begins, and the activity of origin core components is frequently stimulated by association with enhancer or promoter elements [DePamphilis, 1993a,b]. The function of all of these transcription and replication elements depends on binding specific transcription factors or origin recognition proteins.

Whereas the primary function of promoters is generally believed to facilitate assembly of an active initiation complex, the primary function of an enhancer remains controversial. Two models have been suggested: (1) enhancers serve the same function as promoters but use transcription factors that can operate from more distal sites [Carey et al., 1990; Schatt et al., 1990]; and (2) enhancers serve a function distinct from that of promoters; the primary function of enhancers is to relieve repression of weak promoters by chromatin structure [Felsenfeld, 1992] and possibly promoter specific repressors as well [Majumder et al., 1993]. Results described in this essay strongly support hypothesis two. In addition, they reveal that the need for some of the primary components regulating DNA transcription and replication (e.g., enhancers, TATA boxes, replication origins) are acquired at different stages in animal development. In the case of enhancers, their need appears to be linked to changes in chromatin structure [Majumder et

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al., 1993], while in the case of origins, it is linked to nuclear organization [Gilbert et al., 1993].

## SPECIAL FEATURES OF DNA REPLICATION AND TRANSCRIPTION AT THE BEGINNING OF ANIMAL DEVELOPMENT

Since most regulatory components were characterized by studies of differentiated cells and their viruses, the question arises as to whether undifferentiated embryonic cells require the same regulatory components. Studies in non-mammalian systems such as frogs, sea urchin, and fish have led to some surprising observations. For example, unique DNA sequences that normally regulate transcription or replication in somatic cells are often dispensable or ignored when they are injected into oocytes or cleavage embryos. In *Xenopus* oocytes, the majority of RNA polymerase II transcripts frequently are initiated at incorrect sites [Steinbeisser et al., 1988], and transcriptional regulatory components that are required by cells at later stages in development are dispensable in oocytes [Michaeli and Prives, 1987; Green et al., 1987]. Even more striking is the fact that while bidirectional DNA replication is initiated at specific sites in the chromosomes of differentiated mammalian cells [DePamphilis, 1993c], virtually any DNA injected into non-mammalian eggs undergoes semiconservative replication, and early embryos of amphibians and flies recognize at least 5 times more initiation sites than do differentiated cells from the same animals [DePamphilis, 1993a]. These observations suggest that embryonic cells either do not require unique cis-acting sequences to initiate DNA replication or can utilize many different sequences as origins. Alternatively, embryonic cells may not recognize regulatory signals utilized by differentiated cells. For example, polyomavirus can replicate in virtually all cells of the adult mouse, but it cannot replicate in undifferentiated mouse embryonic cells unless its enhancer region is altered.

One possibility is that promiscuity in site selection for DNA transcription and replication is peculiar to non-mammalian embryos since they contain exceptionally high concentrations of maternally inherited mRNA and proteins that permit rapid cell cleavage in the absence of transcription [Davidson, 1986]. For example, a fertilized *Xenopus* egg undergoes 11 cleavages to produce ~4,000 cells in 6 hr, whereas a fertilized mouse egg undergoes only one cleavage event in the first 24 hr (Fig. 1). Thus, the question arises as to what would happen if DNA sequences that normally regulate replication and

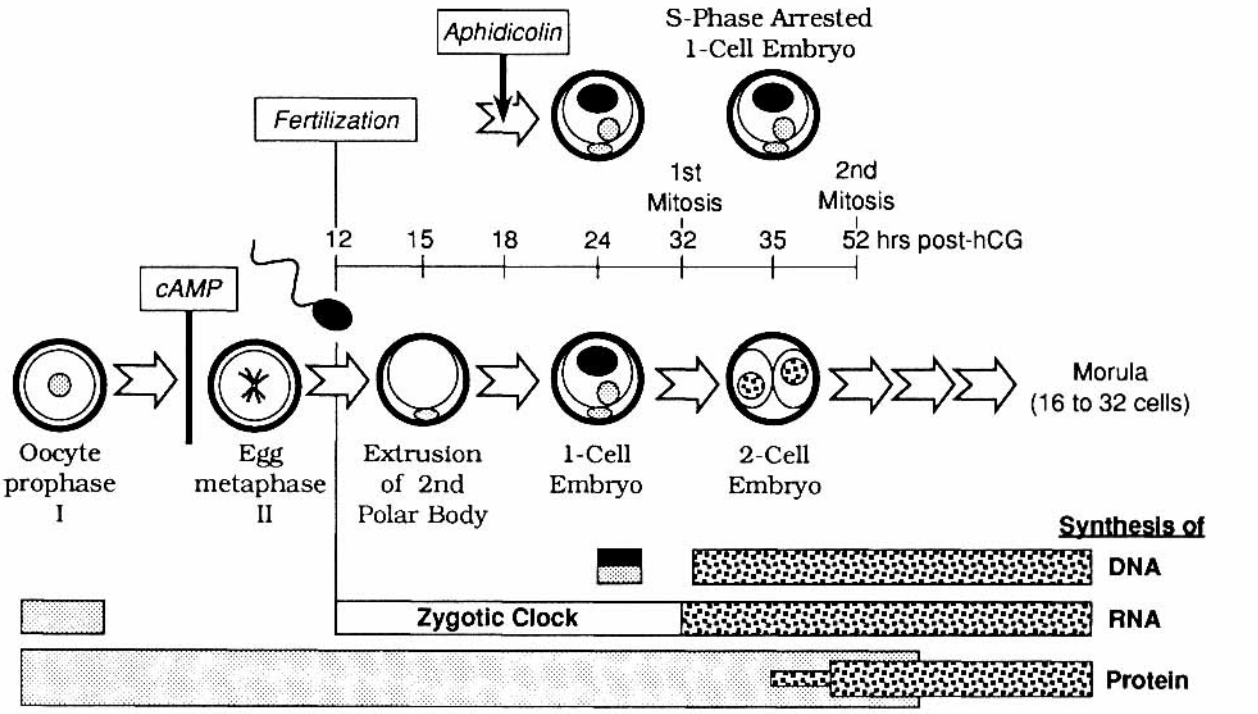


Fig. 1. Mouse preimplantation development and the effects of cAMP and aphidicolin.

transcription in mammalian embryonic or differentiated cells were injected into mammalian oocytes and early embryos. The most well characterized mammalian embryo is the mouse.

## INITIATION OF ZYGOTIC GENE EXPRESSION IN MAMMALS IS CONTROLLED BY A BIOLOGICAL CLOCK

Growing mouse oocytes, arrested at the diplotene stage of the first meiotic prophase, undergo extensive transcription and translation in the absence of DNA replication (Fig. 1) [Wassarman, 1988]. Fully grown oocytes undergo the first meiotic division to become unfertilized eggs arrested in metaphase of the second meiotic division. Transcription then ceases and translation is reduced about 30%. When eggs are fertilized by sperm, they undergo the second meiotic division to produce a 1-cell embryo containing two haploid nuclei, a paternal pronucleus derived from the sperm, and a maternal pronucleus derived from the oocyte. Each pronucleus undergoes DNA replication before merging during the first mitosis to produce a 2-cell embryo containing two diploid zygotic nuclei containing both maternal and paternal chromosomes (Fig. 1). Zygotic gene expression is first detected at the 2-cell stage of mouse development (although expression of injected DNA can be detected in 1-cell embryos [Ram and Schultz, 1993]), and at the 8- to 16-cell stage in other mammals [Telford et al., 1990]. In the mouse, the first α-amanitin sensitive protein synthesis is detected 2–4 hr after cleavage during S-phase, but the major onset of zygotic gene expression begins 8–10 hr later, during G2-phase, concomitant with degradation of maternal RNA. Thus, formation of a 2-cell mouse embryo is accompanied by the onset of zygotic gene transcription and the decline of maternal mRNA-dependent gene expression.

Initiation of zygotic gene expression in the mouse is controlled by a biological clock ("zygotic clock"). Acquisition of a transcriptionally permissive state occurs during the late 1-cell stage of mouse embryogenesis [Latham et al., 1992], but the major transcription-dependent expression of either endogenous genes or injected eukaryotic genes carried on a plasmid begins ~20 hr after fertilization [Martinez-Salas et al., 1989; Conover et al., 1991; Manejwala et al., 1991; Wiekowski et al., 1991]. Normally, this coincides with formation of a 2-cell embryo (~18 hr postfertilization). However, the mechanism that controls the onset of zygotic gene expression depends only on time elapsed, not on DNA replication or morphological development. As a consequence, transcription can occur in 1-cell embryos that have arrested development during the S-phase of their cell cycle, either spontaneously or as a result of DNA synthesis inhibitors. Expression of both cellular and injected genes in these S-phase arrested 1-cell embryos is delayed until the normal time after fertilization when zygotic gene expression begins (Fig. 1).

## REQUIREMENTS FOR DNA TRANSCRIPTION AND REPLICATION AT THE BEGINNING OF MOUSE DEVELOPMENT

To determine the sequence requirements and relative capacities of mouse oocytes and embryos to express genes, the nuclei of oocytes, 1-cell embryos and 2-cell embryos were injected with plasmid DNA containing the firefly luciferase gene driven by one of several viral promoters that is either linked or unlinked to an embryo responsive enhancer placed 600 bp upstream. To determine the ability of oocytes and embryos to replicate DNA, a plasmid containing part or all of the polyomavirus (PyV) origin of replication was coinjected with a plasmid that expressed the polyomavirus large tumor antigen (T-ag) T-ag is the only PyV gene required for viral DNA replication in mouse cells. The critical observations from these injection studies can be summarized as follows:

1. DNA transcription and replication in mouse 2-cell embryos require the same basic regulatory components that have been defined by studies in cultured cells and whole animals. Gene expression requires a eukaryotic promoter and an embryo-responsive enhancer. A prototype for a mammalian embryo-responsive enhancer is the PyV F101 enhancer. It was originally selected for its activity in undifferentiated mouse embryonal carcinoma F9 cells and consists of two tandem TEF-1 DNA binding sites, possibly flanking a third transcription factor binding site not yet identified [Martínez-Salas et al., 1989; Blatt and DePamphilis, 1993; Mélin et al., 1993]. This enhancer is also very active in mouse embryonic stem cells.

The amount of promoter activity and extent of enhancer stimulation depends on the amount of DNA injected (Fig. 2); at low DNA concentrations the PyV F101 enhancer stimulates a weak

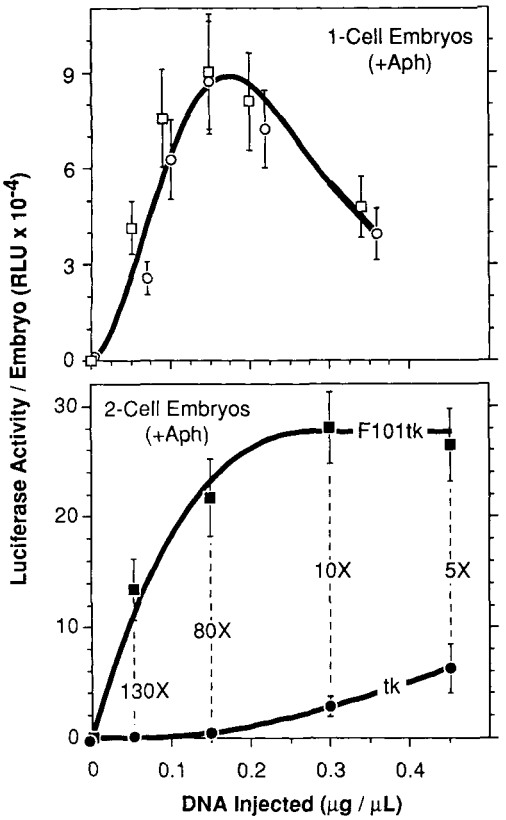


Fig. 2. Expression of luciferase following injection of plasmid DNA ptkluc (○, ●) or pF101tkluc (□, ■) into the paternal pronucleus of a 1-cell embryo **(top)** and into one of the zygotic nuclei of a 2-cell embryo **(bottom)**. Firefly luciferase (luc) is a reporter gene that allows quantitative measurement of promoter/enhancer activity in a single embryo. The herpes simplex virus thymidine kinase (tk) promoter is a well characterized promoter that is utilized by a wide variety of cell types and responds strongly to enhancers. The polyomavirus F101 enhancer (F101) stimulates promoter activity in most mouse cells, particularly in embryonal carcinoma F9 cells, embryonic stem cells and mouse cleavage-stage embryos [Martínez-Salas et al., 1989; Mélin et al., 1993]. Embryos were cultured in aphidicolin to arrest 1-cell embryos at the beginning of S-phase, and 2-cell embryos (which were isolated in their G2-phase) at the beginning of S-phase of the 4-cell stage. Luciferase activity was determined 42 hr postinjection (injected DNA is stable for several days) for 40–60 individual embryos per data point, and the mean value ±SE was expressed as relative light units (RLU) [Miranda et al., 1993]. In the absence of a promoter, luciferase levels were 200 RLU (2-cell embryos) to 500 RLU (1-cell embryos). The extent of enhancer stimulation is indicated at each DNA concentration.

promoter such as the PyV T-ag promoter as much as 350-fold. Similarly, plasmid DNA replication requires a complete viral origin of replication and the origin specific recognition protein. For example, the PyV enhancer stimulates the PyV origin-core 500-fold in the presence of PyV T-ag. Origins of replication in mammalian chromosomes appear to be recognized only when they present in cellular chromosomes organized into nuclei [Gilbert et al., 1993]. Competition experiments between two coinjected plasmids show that transacting cellular proteins are also needed to activate these cis-acting control sequences. In fact, promoters require the same transcription factors in 2-cell embryos that they do in differentiated cells [Majumder et al., 1993], although enhancer activity is strongly dependent on the host cell [Mélin, 1993].

2. Passage of the injected DNA through the first cell division coincides with a general repression of promoter activity that cannot be alleviated, even by an enhancer. When DNA is injected into either pronucleus of 1-cell embryos, and the injected embryos develop to the 2-cell stage or beyond, transcription and replication of the injected DNA is repressed to $<1\%$ of levels observed in 1-cell embryos. Surprisingly, this repression is not alleviated by linking the promoter (or origin of replication) to an embryo-responsive enhancer [Wirak et al., 1985; Wiekowski et al., 1991].

3. The ability of DNA to undergo transcription and replication is not repressed while it remains in the paternal pronucleus of S-phase arrested 1-cell embryos (Fig. 1). Under these conditions, the PyV origin core component alone replicates in the presence of PyV T-ag as efficiently in spontaneously arrested 1-cell embryos as it does when linked to an enhancer and injected into 2-cell embryos [Martinez-Salas et al., 1988]. Likewise, the level of gene expression from DNA injected into arrested 1-cell embryos, with or without an embryo responsive enhancer, is similar to that in 2-cell embryos when the injected DNA contains an enhancer. The enhancer stimulated level of gene expression in 2-cell embryos is never more than three times that observed with DNA injected into 1-cell embryos, and the level of promoter activity from injected 2-cell embryos is $\sim 3\%$ the level of promoter activity from S-phase arrested 1-cell embryos injected with the same amount of DNA (Fig. 2).

These differences between 1-cell and 2-cell embryos are not dependent on either their arrest at S-phase or at mitosis. Two-cell embryos arrested at the beginning of S-phase, that have developed to the 2-cell or 4-cell stage, still require the same embryo responsive enhancer. The enhancer stimulation is 2- to 3-fold greater

in arrested 4-cell embryos than it is in unarrested developing embryos. These differences between 1-cell and 2-cell embryos are also not dependent on the time of injection relative to the cell proliferation cycle, or the developmental history of the embryo [Wiekowski et al., 1991].

In fact, replication and expression of injected DNA is regulated by the same biological controls that govern embryonic development. Plasmid DNA undergoes replication or transcription only when unique eukaryotic regulatory sequences are present and only in cells competent for that function, and genes injected into S-phase arrested 1-cell embryos are not expressed until the "zygotic clock" activates zygotic gene expression (see above). Thus, enhancers appear to provide a unique function in vivo that is dispensable in the paternal nucleus of 1-cell embryos.

There are essentially two ways to explain the lack of need for enhancers in arrested 1-cell embryos to activate either promoters or origins of replication. The first deals with changes in either the amounts or types of transcription factors present. The second deals with changes in chromatin structure or nuclear organization. The following results eliminate the first hypothesis and provide support for a specific role of enhancers in preventing chromatin structure from repressing promoters and origins of replication.

## THE NEED FOR ENHANCERS IS NOT DEPENDENT ON TRANSCRIPTIONAL CAPACITY OR PROMOTER REQUIREMENTS

One explanation for the inability of the Py F101 enhancer to stimulate promoters injected into 1-cell embryos might simply be the absence of appropriate transcription factors required to activate this enhancer. If these proteins were present, then activity in 1-cell embryos might increase to even higher levels than previously observed. To address this question, GAL4:VP16, a fusion of the DNA binding domain from the yeast transcription factor GAL4 and the acidic activation domain from herpes simplex virus (HSV) coat protein 16, was tested for its ability to function in the capacity of a promoter (GAL4 DNA binding sites proximal to a TATA box) and an enhancer (GAL4 DNA binding sites 600 bp upstream from the HSV thymidine kinase (tk) promoter) in both 1-cell and 2-cell embryos [Majumder et al., 1993]. Although GAL4:VP16 is not present in mammalian cells, it strongly stimulates transcription in mammalian cells when GAL4 DNA binding sites are present. When the plasmid containing the GAL4-dependent promoter was coinjected with saturating amounts of an expression vector for GAL4:VP16 protein, the GAL4-dependent promoter was fully active in both 1-cell and 2-cell embryos. In contrast, the GAL4-dependent enhancer stimulated activity only in 2-cell embryos, and the activity observed was equivalent to stimulation of the tk promoter by the embryo-responsive F101 enhancer. Furthermore the failure of the GAL4-dependent enhancer to stimulate the tk promoter in 1-cell embryos was not due to a limited ability of these cells to express luciferase, because an Sp1-dependent promoter produced at least 10 times more luciferase than the tk promoter under the same conditions. Therefore, enhancers serve a function in 2-cell embryos that is not required in 1-cell embryos.

The ability of 1-cell embryos to dispense with enhancers is not related to changes in the requirements for promoter elements. Linker-scanning mutations that inactivate each of the four transcriptional elements in the tk promoter (Sp1-CTF-Sp1-TATA) had the same relative effect on promoter activity in 1-cell embryos as they did in 2-cell embryos and mouse fibroblasts [Majumder et al., 1993], demonstrating that the same promoter elements that are required for transcription under conditions where enhancers stimulate promoter activity (2-cell embryos and fibroblasts) are also required under conditions where enhancers are dispensable (arrested 1-cell embryos). Thus, the need for enhancers does not reflect changes in the utilization of promoter elements.

The ability of 1-cell embryos to dispense with enhancers is not due to an unusually high capacity for utilizing promoters (i.e., transcriptional capacity). If the role of an enhancer is to increase promoter strength by providing additional transcriptional elements, then enhancers would have little, if any, effect in cells with a high transcriptional capacity. In fact, comparing the activities of different promoters of varying strength, in the presence and absence of the embryo-responsive F101 enhancer, reveals that the overall transcriptional capacities of 1-cell and 2-cell embryos are equivalent. Maximum levels of gene expression in 2-cell embryos, however, are realized only with the help of either an enhancer or an exceptionally strong promoter; enhancers have their greatest effect on weak promoters [Majumder et al., 1993].

The general conclusion concerning transcriptional capacity is also evident at the level of a single transcription factor. The tk promoter depends strongly on transcription factor Sp1. Competition experiments for the available Sp1 activity revealed that the amount of Sp1 activity in 2-cell embryos, where enhancers are required to reach full promoter activity, is ~7-fold greater than in arrested 1-cell embryos where enhancers are dispensible [Majumder et al., 1993]. Therefore, the ability of an enhancer to stimulate promoters in 2-cell embryos cannot be explained by a decrease in the amount of a rate-limiting transcription factor that is required to activate the promoter.[1]

## THE NEED FOR ENHANCERS IS TO RELIEVE REPRESSION FROM CHROMATIN STRUCTURE

If the decrease in promoter and origin activity observed in 2-cell embryos cannot be accounted for by changes in the types or amounts of transcription factors, it must result from repression. Since this repression affects a diverse range of promoter and origin sequences that bear little homology and that interact with different initiation factors, repression likely results from chromatin structure.

Transcriptionally active eukaryotic genes are associated with acetylated core histones [Grunstein, 1990; Turner, 1991]. Butyrate rapidly blocks histone deacetylase, thus increasing the fraction of acetylated core histones and allowing greater access of various protein to DNA. Part of this access is achieved by releasing negative supercoils previously constrained into a closed chromatin loop and part by reducing the ability of nucleosomes to interact with histone H1. These are properties of actively transcribed chromatin. In this way, butyrate can stimulate the expression of cellular or plasmid encoded genes.

DNA injected into mouse embryos, like endogenous DNA, is assembled into nucleosomes [Martinez-Salas et al., 1989] but, unlike endogenous DNA, plasmid expression vectors do not replicate because they lack a functional origin [DePamphilis et al., 1988a,b]. Butyrate stimulates the activity of promoters injected into 2-cell embryos, restoring it to the level observed in paternal pronuclei of 1-cell embryos and thereby alleviating the need for enhancer activity in vivo [Wiekowski et al., in press]. In the presence of butyrate, the F101 enhancer stimulates promoters only 2- or 3-fold compared to about 100 fold in the absence of butyrate. Butyrate does not stimulate promoters injected into the paternal pronucleus of arrested 1-cell embryos but does stimulate promoters injected into maternal pronuclei, strongly suggesting that the effects of butyrate on promoter activity do not result from stimulating synthesis of transcription factors. Newly synthesized transcription factors should act on both pronuclei. Similar results were observed in the presence of Gal4:VP16 and a GAL4-dependent enhancer [Majumder et al., 1993]. Furthermore, butyrate also has no effect on protein synthesis from maternally inherited mRNA, and can stimulate only those endogenous genes that are transcriptionally active. Thus, the need for enhancers in 2-cell embryos is most likely to alleviate chromatin-mediated repression (Fig. 3). This does not exclude a secondary role of enhancers in facilitating formation of the initiation complex. Such a role could account for the 2- to 3-fold stimulation of promoters in mouse 2-cell embryos by the F101 enhancer above the level observed in 1-cell embryos, as well as low levels of enhancer activity sometimes observed in vitro in the absence of chromatin assembly.

In general, enhancers have little effect on promoter or origin activity unless the DNA is organized into chromatin [discussed in Majumder et al., 1993]. The small effects of enhancers observed in vitro are consistent with simply changing the strength of the promoter, rather than employing the primary function of enhancers in vivo which is to relieve repression from chromatin structure. GAL4:VP16 was reported to stimulate transcription in cell extracts when Gal4 DNA binding sites were placed distal (enhancer function), as well as proximal (promoter function), to the TATA box, but transcription was reduced 27-fold as the distance between Gal4 DNA binding sites and the TATA box was increased from 102 bp to 1318 bp [Carey et al., 1990]. Moreover, the ability of distal Gal4 DNA binding sites to stimulate transcription was not observed by others unless the DNA template was packaged into histone H1-containing chromatin [Laybourn and Kadonaga, 1992], suggesting that Gal4 DNA binding sites acting as an enhancer at 600 bp upstream of the tk promoter will stimulate transcription only under conditions where a repressive chromatin structure is formed.

[1]A typographical error on page 1137, column 1, line 27 of Majumder et al. [1993] says "*can* be explained . . ." instead of "cannot be explained. . . ."

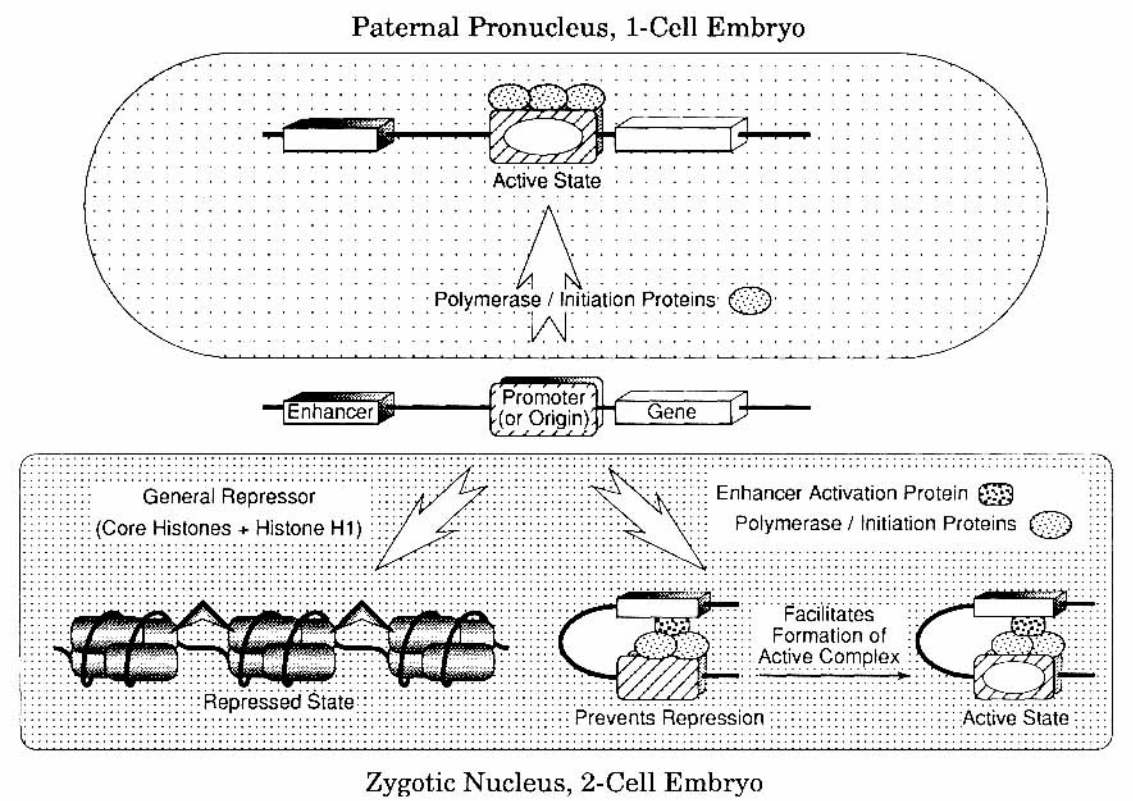


Fig. 3. The primary role of enhancers is to prevent repression of promoters and origins of DNA replication by chromatin structure.

Experiments in which DNA is injected into cell nuclei are analogous to experiments in which DNA is added to cell extracts that can assemble chromatin. In both cases, transcription or replication factors must compete with histones for binding to DNA. Nucleosome assembly can repress both transcription and replication by interfering with either the activity of transcription or replication proteins [Workman and Buchman, 1993]. When nucleosome spacing on in vitro assembled templates approximates the spacing of nucleosomes in vivo, then most of the transcriptional repression ( ~ 100-fold) results from condensation of chromatin by histone H1 [Laybourn and Kadanoga, 1992]. Thus, paternal pronuclei in arrested 1-cell embryos behave as though they do not assemble DNA into mature H1-containing chromatin, whereas zygotic nuclei in 2-cell embryos behave as though they do.

## ABSENCE OF REPRESSION IS UNIQUE TO PATERNAL PRONUCLEI IN 1-CELL EMBRYOS

Maternal pronuclei in oocytes, 1-cell embryos and parthenogenetically activated eggs exhibit from 6% to 18% of the promoter activity observed in paternal pronuclei of arrested 1-cell embryos [Wiekowski et al., in press; S. Majumder, unpublished results]. Since butyrate stimulated promoter activity in maternal pronuclei to a level similar to that observed in paternal pronuclei, the decreased promoter activity in maternal pronuclei is most likely due to repression by chromatin structure. Therefore, one would expect that an appropriate enhancer would stimulate promoters in maternal pronuclei. The F101 enhancer does not stimulate promoter activity in maternal pronuclei, but the GAL4-dependent enhancer does, although stimulation of promoter activity in oocytes and 1-cell embryos (4- to 5-fold) is significantly less than in 2-cell embryos ( ~ 35-fold) [S. Majumder, unpublished results]. Thus, repression of promoter activity exists in maternal pronuclei prior to the 2-cell stage, but the striking ability to relieve this repression with enhancers first appears in 2-cell embryos concurrent with the onset of zygotic gene expression.

Formation of 2-cell embryos is accompanied by a strong repression of promoters regardless of the parental origin or ploidy of its nuclei. Promoters are repressed to a similar extent in 2-cell embryos that were constructed with hap-

loid or diploid nuclei containing either maternal, paternal or zygotic nuclei [Wiekowski et al., in press]. In each case, an injected promoter was stimulated by either the F101 enhancer or butyrate, consistent with the concept of repression due to chromatin structure formed on the DNA template. Nuclear origin, and to a lesser extent chromosome ploidy, did, however, effect the transcriptional capacity of 2-cell embryos (zygotic nuclei > maternal nuclei > paternal nuclei; diploid > haploid), suggesting that the levels of transcription factors available at the onset of zygotic gene expression are greatest in embryos produced from fertilized eggs. These differences in the transcriptional capacity of 2-cell embryos with nuclei of different parental origin and ploidy paralleled the ability of such embryos to support mouse development [Surani et al., 1986].

## CHANGES IN HISTONE SYNTHESIS AT THE BEGINNING OF MOUSE DEVELOPMENT ARE CONSISTENT WITH CHANGES IN THE REQUIREMENTS FOR TRANSCRIPTION

Based on the effects of butyrate, one might expect differences in the acetylated state and histone composition of chromatin assembled in oocytes, 1-cell and 2-cell embryos. In fact, the pattern of histone synthesis and histone H4 acetylation at the beginning of mouse development [M. Wiekowski, unpublished results] is consistent with this hypothesis, although the actual composition of plasmid chromosomes has not yet been determined.

All five major histones are synthesized in mouse oocytes, and the pattern of histone synthesis is similar to that of mouse fibroblasts. The acetylated state of histone H4 is primarily zero or one in these cells. In contrast, synthesis of only histones H3 and H4 resumes in early 1-cell embryos (prior to pronuclear formation); synthesis of histones H2A, H2B and H1 first appears in late 1-cell embryos. Nascent histone H4 in 1-cell embryos is predominantly diacetylated, and the amount of hyperacetylated H4 detected by antibodies is the same in paternal and maternal pronuclei. The pattern of histone synthesis in 2-cell embryos is indistinguishable from the pattern in late 1-cell embryos. Histone synthesis in 1-cell and 2-cell embryos is α-amanitin insensitive and therefore appears to be translated from maternally inherited mRNA. Somatic histone H1 (and presumably core histones as well) is not detected until the 4-cell stage [Clarke et al., 1992]. Thus, chromatin assembled in 1-cell embryos may be deficient in H2A, H2B, and H1 and contain hyperacetylated histones, while chromatin assembled in 2-cell embryos should be assembled into mature chromatin, except that it lacks hyperacetylated histones.

These changes in maternal histone synthesis alone cannot explain the dramatic repression of promoter activity in 2-cell embryos, because the same changes occurred in S-phase arrested 1-cell embryos where repression does not appear in paternal pronuclei. Moreover, repression does not occur from simply combining the contents of maternal and paternal pronuclei. Repression occurred in all 2-cell embryos, regardless of nuclear origin or ploidy, and repression occurred when 1-cell embryos were arrested with cytochalasin D in G2-phase before the two pronuclei merged [Martinez-Salas et al., 1989; S. Majumder, unpublished results]. One possibility is that the first round of DNA replication in mammals triggers formation of some factor that is required for chromatin structure to mediate repression, possibly by post-translational modification of one or more histones.

## SIGNIFICANCE OF TRANSCRIPTIONAL REPRESSION TO EARLY ANIMAL DEVELOPMENT

Two phases of transcriptional inhibition occur at the beginning of animal development: inhibition of transcription by the zygotic clock whose mechanism is unknown, and repression of promoters by chromatin structure. The purpose of the zygotic clock could be to delay transcription until chromatin remodeling is completed and paternal and maternal genomes are returned from a post-meiotic state to one in which the basal level of a gene's activity is repressed. In this way, the ability of enhancers to relieve this repression could provide a specific mechanism by which to activate transcription of genes at specific times during development.

The difference in repression of injected promoters in maternal and paternal pronuclei may be traced to their respective germ cells. The maternal genome is packaged into chromatin in oocytes. Oocytes contain a normal complement of core histones, and oocytes efficiently assemble injected DNA into chromatin that represses promoter activity unless treated with butyrate. However, the paternal genome arrives packaged in protamines that are then replaced with histones provided by the egg, regardless of whether or not DNA replication occurs [Zirkin et al.,

1989]. Since paternal pronuclei neither repress promoter activity nor respond to butyrate, remodeling of the paternal genome may utilize hyperacetylated core histones to facilitate chromatin assembly. Thus, the fact that paternal and maternal genomes are assembled into chromatin under different conditions could account for their different responses to injected DNA. Since expression of some genes is determined by whether they reside on chromosomes inherited from the mother or father ("genomic imprinting") [Hall, 1990], the apparent difference in chromatin structure assembled in paternal and maternal pronuclei of 1-cell embryos and subsequent passage through the first mitosis could result in imprinting of genes by creating differences in their accessibility to transcription factors and methylation enzymes.

Enhancers are the first example of a basic regulatory component of transcription that is acquired at a specific stage in mammalian development: formation of a 2-cell mouse embryo. A second example has recently been discovered [Majumder and DePamphilis, in press]. The HSV thymidine kinase promoter required its TATA box only after mouse cells began to differentiate and then only when promoter activity was stimulated by either an enhancer or transactivator. Disruption of the TATA box by a site specific mutation did not reduce tk promoter activity, either linked or unlinked to the embryo-responsive PyV F101 enhancer, when introduced into early mouse embryos from 1-cell to 8-cell stage in development or into undifferentiated mouse embryonic stem cells. Similarly, tk promoter activity was dependent on its TATA box in mouse oocytes, a terminally differentiated cell that contains its own transactivator. These results identify conditions under which a TATA box element is needed for promoter activity, and provide an example of a basic transcriptional element whose function is developmentally acquired. Moreover, they link the role of a TATA box to the function of enhancers and transactivators.

The same mechanisms of transcriptional control that initiates mouse development seem also to occur in other animals. In mammals other than mice, transcription is delayed until the 4-cell to 16-cell stage [Telford et al., 1990], presumably by the same zygotic clock mechanism. In rabbits, transcription is delayed until ~33 hours post-fertilization (corresponding to the 8 to 16-cell stage), but enhancers are not required until formation of morula [Delouis et al., 1992], suggesting that the appearance of repression coincides with the onset of zygotic gene transcription. The S-phase of a 2-cell mouse embryo appears equivalent to the 6th cleavage stage in *Xenopus* where synthesis of heterogeneous, nonribosomal mRNA is first detected, and the G2-phase of a 2-cell mouse embryo appears equivalent to the 12th cleavage stage in *Xenopus* where the major onset of RNA polymerase II and III transcription occurs, that is, the midblastula transition (MBT) [Kimelman et al., 1987; Shiokawa et al., 1989]. The activity of promoter/enhancer sequences injected into *Xenopus* eggs is generally delayed until the MBT, although they appear to exhibit a low but constant rate of gene expression per cell prior to the MBT [Shiokawa et al., 1990]. Activation of transcription at the MBT can require specific enhancers [Krieg and Melton, 1987], analogous to the need for an enhancer to activate promoters in 2-cell mouse embryos. The MBT also marks the appearance of histone H1 mediated repression of oocyte specific genes such as 5S RNA [Wolffe, 1989; Ohsumi and Katagiri, 1991], analogous to the repression observed upon formation of 2-cell mouse embryos.

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