The role of chromatin in the establishment of enhancer function during early mouse development

Review Article

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Summary

In mammals, enhancers are believed to stimulate transcription from RNA polymerase II promoters primarily by relieving their chromatin-mediated repression. Interactions responsible for enhancer function are developmentally acquired. Factors responsible for this repression are not present in the paternal pronuclei of 1-cell embryos, making them impervious to enhancer function. Although such chromatin-mediated repression is observed in oocytes, maternal pronuclei of 1-cell embryos, and the 2-cell embryos, the enhancer function first appears in 2-cell embryos, a stage in development that corresponds to the beginning of major zygotic gene expression (ZGE). The lack of enhancer function prior to 2-cell stage is also not due to the absence of specific activation proteins, but appears to be due to the absence of an essential coactivator activity. The coactivator activity first appears in 2-cell embryos, and is required for enhancers driven by different classes of transcription factors. The absence of the coactivator activity and the corresponding enhancer function appears to be unique to oocytes and 1-cell embryos, suggesting that it provides a safeguard against premature activation of genes prior to ZGE.

I. Introduction

Transcription by RNA polymerase II is controlled primarily by two elements: promoters and enhancers. Promoters determine where transcription begins, and they function upstream and proximal to the initiation site. Enhancers stimulate weak promoters in a tissue specific manner, and they function distal to the initiation site from either an upstream or downstream position. Enhancers can also function as components of origins of DNA replication (DePamphilis 1997) where their activity depends on binding specific transcription factors that can interact with replication proteins (He et al. 1993). However, whereas the primary function of promoters and replication origins is to facilitate assembly of an active initiation complex, the primary function of enhancers remains controversial. Two models are generally considered. In the first model, enhancers are simply extensions of promoters; enhancers and promoters both facilitate formation of an active transcription complex with enhancers providing additional transcription factors that can function at a greater distance from the mRNA start site (Carey et al. 1990; Schatt et al. 1990). This model is encouraged by the fact that the same transcription factors frequently can function as components of either promoters or enhancers. In the second model, the primary role of an enhancer is distinct from that of a promoter; whereas promoters facilitate formation of an active complex, enhancers stimulate weak promoters by relieving chromatin mediated repression of promoters, a process that might involve histone acetylation (Grunstein 1997; Pazin and Kadonaga 1997; Roth and Allis 1996; Vermaak and Wolffe 1998; Wolffe and Pruss 1996). Support for this model comes from analyses of gene expression and of chromatin structure in mouse preimplantation embryos and transcription in cell free systems.
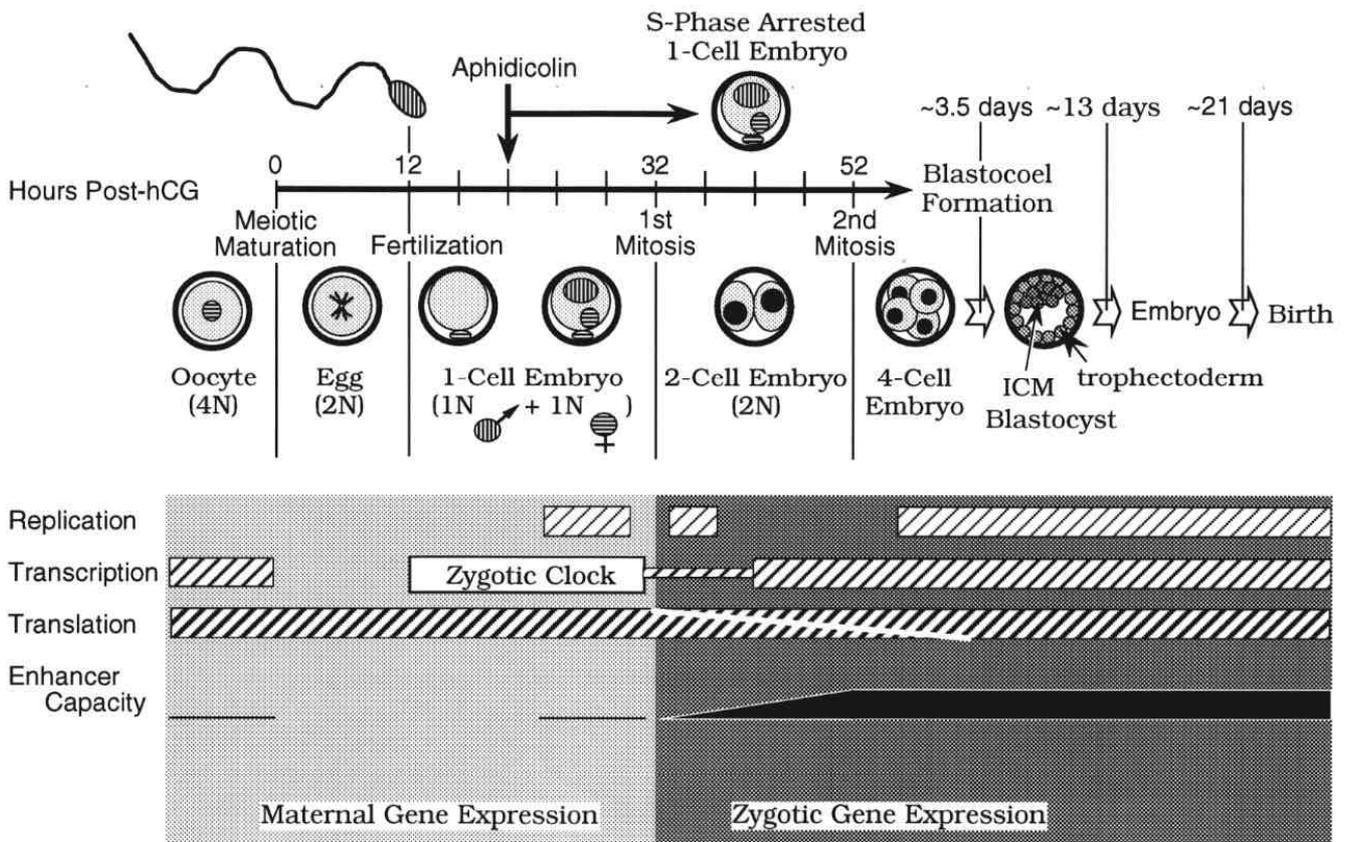
Figure 1. Schematic representation of events at the beginning of mouse development. **Upper Panel:** Morphological and cell cycle events are indicated as a function of time after injection of human chorionic gonadotropin (post-hCG), a hormone that stimulates ovulation. The paternal pronucleus is indicated by vertical striations, oocyte and maternal pronucleus are denoted by horizontal striations, and the zygotic nuclei are represented by filled circles. Addition of aphidicolin to 1-cell embryos prior to the appearance of pronuclei insures their morphological arrest as they enter S-phase. **Lower Panel**: Events in gene expression are divided into two phases: maternal and zygotic gene expression. Periods of DNA replication, transcription and mRNA translation are indicated by bars. The ability of oocytes and embryos to utilize enhancers encoded in plasmid DNA injected into cell nuclei is indicated as the general capacity of enhancers to stimulate promoter activity. The resumption of transcription in embryos is delayed by a time-dependent biological clock mechanism (zygotic clock).

II. Early mouse development

Relevant features of early mouse development are diagrammed in **Figure 1**. Growing oocytes, arrested at diplotene of the first meiotic prophase, transcribe and translate many of their own genes. Transcription stops during meiotic maturation, when unfertilized eggs arrest in metaphase of the second meiotic division. Fertilization triggers completion of meiosis and formation of a 1-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 2-cell embryo containing two diploid "zygotic" nuclei, each with a set of paternal and a set of maternal chromosomes.

While translation of maternally inherited mRNAs occurs continuously in mature eggs and 1-cell embryos, 1-cell embryos transcribe endogenous genes at a very low rate (Aoki et al. 1997). They can also transcribe microinjected plamids (Ram and Schultz 1993), with the male pronucleus being 4 to 5 more active than the female (Henery et al. 1995; Majumder 1997; Wiekowski et al. 1993).

Major zygotic gene activation (ZGA; -amanitin sensitive protein synthesis) begins in 2-cell embryos. Prior to S-phase only a small number of proteins are expressed (Christians et al. 1995; Davis et al. 1996); after S-phase both the number and overall rate of protein synthesis

increases significantly (Latham et al. 1991). Concurrent with these events is a rapid degradation of maternally inherited mRNAs, reflecting a switch from maternal to zygotic control of embryonic development (Telford et al. 1990)

If 1-cell embryos are arrested in S-phase using inhibitors of DNA replication, they remain morphologically 1-cell embryos, but ZGA still occurs at the same time post-fertilization that it normally occurs in developing embryos (i.e. the time when 2-cell embryos appear) (Aoki et al. 1997; Conover et al. 1991; Majumder and DePamphilis 1995; Wiekowski et al. 1991). Therefore, initiation of ZGA is governed by a biological clock ("zygotic clock", **Fig. 1**) that delays transcription until a specified time after fertilization. Other mammals also exhibit a zygotic clock, although the developmental stage at which transcription begins is species specific. Zygotic gene expression begins in at the 2-cell stage in mice and hamsters, the 4-cell stage in pigs, the 4 to 8-cell stage in humans, and the 8 to 16-cell stage in sheep, rabbits and cows (Braude et al. 1988; Ferrer et al. 1995; Schultz 1993; Seshagiri et al. 1992).

III. Injection of DNA as a method for studying DNA transcription and replication in early mammalian embryos

The fact that mammalian embryos are available in limited quantities places a serious roadblock in front of any effort to identify cis-acting sequences and trans-acting factors that are required for DNA transcription or replication at the beginning of mammalian development. One solution to this problem has been to inject plasmid DNA into the nuclei present in oocytes, 1-cell embryos, or 2-cell embryos (**Fig. 1**) and then identify sequences that are required to either replicate the plasmid or express an encoded reporter gene. These transient assays, like those used following transfection of cultured mammalian cells, reveal the capacity of cells to replicate or express genes, their ability to respond to specific cis-acting sequences, and their ability to utilize specific trans-acting factors provided by co-injecting expression vectors (Majumder 1997). A variety of promoters and enhancers or transactivators have been examined in mice or rabbits. These include promoters for the early genes of SV40 (Bonnerot et al. 1991; Chalifour et al. 1987; Chalifour et al. 1986; Delouis et al. 1992; Ram and Schultz 1993) and polyomavirus (Melin et al. 1993), herpes simplex virus thymidine kinase gene (Majumder et al. 1993; Martinez-Salas et al. 1989; Wiekowski et al. 1991), adenovirus EIIa (Dooley et al. 1989), mouse ZP3 (Lira et al. 1990; Millar et al. 1991), hypoxanthine phosphoribosyl transferase, -actin and hydroxymethyl glutaryl CoA reductase (Bonnerot et al. 1991; Delouis et al. 1992; Vernet et al. 1992), TATA-box and Sp1-dependent promoters (Majumder et al. 1993). The most active transcription factor identified so far in growing oocytes (Chalifour et al. 1987; Chalifour et al. 1986) to cleavage-stage embryos (Majumder et al. 1993) is Sp1.

The responses of DNA injected into embryonic cell nuclei reflect the normal conditions at the beginning of mammalian development, because the injected DNA responds to the same signals that regulate endogenous DNA replication and gene expression. Injected DNA undergoes replication and transcription (**i**) only when unique eukaryotic regulatory sequences are present, and (**ii**) only in cells that are competent for that function (Majumder and DePamphilis 1994). For example, mouse oocytes are arrested in meiosis and therefore do not replicate their own DNA. Accordingly, oocytes do not replicate plasmid DNA injected into their nucleus, even if the injected DNA contains a viral origin and is provided with the appropriate viral replication proteins (Chalifour et al. 1986; Wirak et al. 1985). In contrast, plasmid DNA does replicate when injected into 1-cell or 2-cell embryos that undergo S-phase (Wirak et al. 1985), but only when the plasmid contains a functional viral origin and only when the cognate origin recognition protein is present (Martinez-Salas et al. 1988).

On the other hand, oocytes do express some of their own genes, and they also can express genes encoded by plasmids if an appropriate promoter is present (Chalifour et al. 1987; Dooley et al. 1989). For example, the oocyte-specific promoter for zona pellucida protein-3 provides oocyte-specific expression when present on injected plasmid DNA (Lira et al. 1990; Millar et al. 1991; Schickler et al. 1992). DNA transcription can be analyzed in 1-cell embryos that are arrested as they enter S-phase by including aphidicolin in the culture medium, because the zygotic clock still triggers zygotic gene activation at the normal time post-fertilization. Thus, genes injected into a pronucleus of S-phase−arrested 1-cell embryos remain inactive for as long as 20 hours until the first zygotic genes are expressed (Martinez-Salas et al. 1989; Wiekowski et al. 1991). In contrast, plasmid-encoded genes are expressed immediately when injected into 2-cell embryos that are actively expressing their own cellular genes, regardless of whether these embryos are cultured in the presence or absence of aphidicolin. Thus, we conclude that the response of plasmid DNA injected into mammalian oocytes and embryos is not an artifact of the experimental protocol, but accurately reflects the cell's capacity for carrying out transcription or replication *in vivo*.

IV. Enhancers relieve promoter repression: a lesson from 1-cell and 2-cell embryos

Microinjection experiments using plasmid DNA containing a reporter gene linked to a promoter and/or an enhancer revealed that promoters injected into the paternal pronucleus of S-phase−arrested 1-cell embryos are highly active and their activity is not increased by linking them to enhancers. In contrast, promoters injected in the zygotic nuclei of 2-cell embryos are strongly repressed and this repression can be relieved by linking them to an embryo specific enhancer. For example, the F101 enhancer can stimulate various RNA pol II promoters from 20 fold to more than 300 fold in 2-cell embryos depending on promoter strength and the amount of DNA injected, but does not stimulate the same promoter in 1-cell embryos (Majumder and DePamphilis, 1995).

Why are the activities of promoters so much lower in 2-cell embryos than in the paternal pronucleus of 1-cell embryos? It is not due to changes in the requirements for transcription factors, because site specific mutations affecting individual transcription factor binding sites in the tk promoter show that 1-cell and 2-cell mouse embryos utilize the same promoter elements (Majumder et al. 1993). It is also not due to a decreased ability to utilize promoters, because arrested 2-cell and 4-cell embryos are as effective as arrested 1-cell embryos in utilizing a variety of promoters, if the promoters are linked to the F101 PyV enhancer (Majumder et al. 1993). Therefore, the transcriptional capacity of 2-cell embryos is equivalent to that of 1-cell embryos. This is more directly seen by comparing the amount of a specific transcription factor, such as Sp1, that limits the activity of the tk promoter in both 1-cell and 2-cell embryos. Although the tk promoter is at least 20-fold more active in the paternal pronucleus of arrested 1-cell embryos than in the zygotic nuclei of arrested 2-cell and 4-cell embryos, the amount of Sp1 in 2-cell embryos is about 6-fold greater than in 1-cell embryos (Majumder et al. 1993; Worrad et al. 1994). Therefore, the reduction in the capacity of embryos to utilize promoters observed upon formation of a 2-cell embryo must result from the appearance of a repressor rather than the loss of positively acting transcription factors.

This repression does not likely result from proteins that bind to specific sequences, because it affects different promoters and replication origins that bear little sequence homology and that interact with different initiation factors. This repression also does not depend on the presence of a zygotic nucleus, because it occurs in 2-cell embryos constructed to contain diploid or haploid maternal or paternally derived nuclei (Wiekowski et al. 1993) and in paternal pronuclei that have been transplanted to 2-cell embryos (Henery et al. 1995). Instead, this repression appears to be mediated by changes in chromatin structure that result, at least in part, from factors that are present in the cytoplasm of 2-cell embryos but absent from the cytoplasm of 1-cell embryos.

Treatment of 2-cell embryos with butyrate or trichostatin strongly stimulates promoter activity, relieving repression and reducing the need for enhancers (Majumder et al. 1993; Wiekowski et al. 1993). Butyrate has been found to stimulate promoters in mammalian cells by altering the acetylated state of chromatin structure (Grunstein 1997; Kamakaka and Thomas 1990; Tazi and Bird 1990). Butyrate rapidly blocks histone deacetylase, thus increasing the fraction of acetylated core histones that makes the DNA more accessible to transcription factors and reduces the ability of nucleosomes to interact with histone H1 (Turner 1991). Two lines of evidence confirm that the effect of butyrate on relieving repression is at the level of chromatin structure rather than increasing the levels of transcription factors. Butyrate has opposite effects on maternal and paternal pronuclei in 1-cell embryos (discussed below), and does not change the pattern of endogenous protein synthesis in 1-cell and 2-cell embryos (Wiekowski et al. 1993). The effect of butyrate/trichostatin also correlates with composition and modification status of histones present in the embryos as discussed below.

Thus, taken together, these results indicate that the primary function of enhancers is to relieve promoter repression that is observed in 2-cell embryos, and not in 1-cell embryos. Enhancers presumably perform this function by preventing chromatin structure from interfering with assembly of an initiation complex. To accomplish this task, transcription factors that activate enhancers must fulfill two criteria: (**i**) they must successfully compete with chromatin structure, and (**ii**) they must interact with at least one of the transcription factors that constitute a promoter. Transcription factors that cannot compete with chromatin structure would constitute weak promoters that require an enhancer for full activity, whereas when transcription factors that can compete with chromatin constitute part or all of a promoter, the need for an enhancer to activate that promoter would decrease accordingly. This model 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 PyV enhancer above the level observed in 1-cell embryos (Majumder et al., 1993) as well as low levels of enhancer activity sometimes observed *in vitro* in the absence of chromatin assembly.

V. Enhancer function requires a specific coactivator: a lesson from oocytes and 2-cell embryos

In summary, the studies discribed above revealed that transcription promoters and replication origins introduced into the nuclei of 2-cell mouse embryos undergo repression, regardless of the origin or ploidy of the nucleus, and regardless of whether or not cells are arrested in S-phase or allowed to continue cell division. Furthermore, this repression can be relieved either by linking the promoter or origin-core to an embryo responsive enhancer (e.g. F101 PyV enhancer), providing a general transactivator (e.g. HSV ICP4 protein), or by treating the cells with butyrate. Surprisingly, the same DNA sequences are not repressed when injected into the paternal pronucleus of S-phase arrested 1-cell embryos; their activities are high and they are not stimulated further by enhancers, transactivators or butyrate. Since transplantation of the injected paternal pronucleus to a 2-cell embryo returns the injected promoter to a repressed state that can be relieved by enhancers, the absence of repression is not unique to paternal pronuclei, but to the cytoplasm of 1-cell embryos. The environment of the paternal pronucleus in a mouse 1-cell embryo is analogous to cell free systems in which enhancers are no longer needed to activate promoters or replication origins unless the DNA substrate is first assembled into chromatin. Thus, the question remained: what happens when the same sequences are injected into maternal pronucleus of a 1-cell embryo and its precursor, the maternal nucleus (germinal vesicle) of an oocyte?

Since oocytes do not replicate DNA, only promoters were injected into the maternal nucleus of an oocyte, parthenogenetically activated egg, or fertilized egg (1-cell embryo). Under these conditions, promoters are repressed from 80 to 96% relative to the paternal pronucleus of S-phase arrested 1-cell embryos (**Table 1**), (Majumder et al. 1993; Majumder et al. 1997; Martinez-Salas et al. 1989; Wiekowski et al. 1993). As with 2-cell embryos, repression can be relieved by treating cells with butyrate, raising the level of promoter activity in maternal nuclei to that observed in paternal pronuclei. This suggests that the mechanism of repression in maternal nuclei of oocytes and 1-cell embryos is the same as in 2-cell embryos. In fact, the composition of nascent histones in mouse oocytes are indistinguishable from those in mouse fibroblasts, and the slight increase of promoter activity observed in maternal pronuclei (**Table 1**) corresponds to increased hyperacetylation of histone H4 (Adenot et al. 1997; Wiekowski et al. 1997). Since the cytoplasm of 1-cell embryos does not contain repressor activity, maternal pronuclei must retain some of the repressor that is produced in oocytes. This may be histone H1 that is associated with cellular chromatin, but can transfer easily to chromatin assembled onto plasmid DNA. However, in contrast to 2-cell embryos, linking the promoter to the F101 enhancer could not relieve repression of promoter activity in the maternal nuclei of oocytes and 1-cell embryos. Therefore, some factor is missing in oocytes and 1-cell embryos that is required for enhancer function.

This missing factor is not the transcription factor that must bind to the enhancer sequence, because oocytes and 1-cell embryos cannot utilize enhancers even when the appropriate enhancer activation protein is provided. In the presence of sufficient GAL4-VP16 protein to drive a GAL4-dependent promoter at its maximum rate, a GAL4-dependent enhancer located 600 bp upstream of the tk promoter stimulates promoter activity only 1.5-fold in the maternal nucleus of oocytes, 2-fold in the maternal pronucleus and none in the paternal pronucleus of S-phase arrested 1-cell embryos. In contrast, the same enhancer stimulates promoter activity at least 30-fold when injected into 2-cell embryos in the presence of saturating amounts of GAL4-VP16 (**Table 1**) (Majumder et al. 1993; Majumder et al. 1997). Therefore, the missing factor is a co-activator protein(s) that mediates the activity of GAL4:VP16 protein with the transcription complex that binds to the promoter (**Fig. 2**). This co-activator protein is specific for enhancer function, because the same series of Gal4 DNA binding sites located proximal to a TATA box (i.e. Gal4-dependent promoter) functions efficiently under all conditions. mRNA isolated from mouse embryonic stem (ES) cells and preinjected into the cytoplasm of mouse oocytes, was able to confers enhancer function. ES cells, like cleavage stage embryos, efficiently utilize the F101 enhancer (Melin et al., 1993), and therefore provided a convenient source of mRNA encoding the putative enhancer coactivator. Whether this activity is brought in by a single molecule or a family of molecules is not known However, our preliminary experiments show that a fraction of the nuclear extract from HeLa cells can restore enhancer activity in oocytes (Rastelli, Zhao and Majumder, unpublished observation). It appears that the coactivator activity mediates protein-protein interaction between factors bound at the enhancer site and the transcription complex bound at the promoter site. Since the chromatin structure and enhancer function are intimately connected, the coactivator activity might act by remodeling chromatin structure (Felsenfeld 1996; Kingston et al. 1996; Pazin and Kadonaga 1997; Struhl 1998).

VI. The appearance of promoter repression correlates with changes in chromatin structure

Repression of promoter activity correlates with a decrease in histone H4 hyperacetylation and the concurrent appearance of histone H1 and H2A and H2B. Metaphase II oocyte chromatin and sperm chromatin do not contain hyperacetylated H4 histones, as revealed by antibody staining

Table 1. Properties of mouse oocytes and preimplanation embryos that affect gene expression.

Stage	Nuclear origin	Promoter repression	Chromatin hyperacetilation	Histone H1,H2A and H2B synthesis	Enhancer stimulation	Putative enhancer-specific coactivator
Oocyte	Maternal	+++	-	++	-	-
1-cell embryo	Maternal	+	+ early	-	-	-
1-cell embryo	Paternal	-	++ early	-	-	-
2-cell embryo	Zygotic	++	+/-	+	+	+
4-cell embryo	Zygotic	+++	-	++	++	++

This table summarizes all the data on oocyte and preimplantation embryo properties that are relevant to explain the difference in transcription activity among the various stages.

(Adenot et al. 1997), or by labeling of nascent H4 (Wiekowski et al. 1997). Adenot et al. showed that upon sperm entry and throughout most of G1, the paternal chromatin in 1-cell embryos has higher level of hyperacetylated H4. Since transcriptionally active eukaryotic genes are associated with acetylated core histones (Turner 1991), the authors propose that hyperacetylated paternal chromatin can preferentially recruit transcription factors at this stage to explain the higher promoter activity. But, the levels of hyperacetylated H4 are similar in both pronuclei during S/G2 (Adenot et al. 1997) and remain high in 2 cell embryos (Wiekowski et al. 1997) and therefore cannot explain the promoter repression that occurs with the formation of the 2 cell embryos. However, the beginning of promoter repression also correlates with the beginning of histones H1, H2A and H2B synthesis in late 1-cell embryos (Wiekowski et al. 1997). The synthesis of these histones is independent of DNA replication, DNA transcription and cell cleavage indicating that they are translated from maternally inherited mRNAs. Antibodies directed against somatic H1 do not detect any H1 until the late 4-cell stage (Clarke et al. 1992), suggesting that this early form of histone H1 may represent a novel histone H1 subtype as seen in other organisms (Ohsumi and Katagiri 1991; Smith et al. 1988). Thus, it appears that promoter repression correlates with the presence of histone H1 and the absence of acetylation (other modification?) of core histones (**Table 1**).

Studies on promoter activity *in vitro* further support the hypothesis that the primary role of enhancers is to prevent repression by chromatin structure. Enhancers have little, if any, effect on DNA replication (Prives et al. 1987) or transcription discussed in (Majumder et al. 1993) when they are assayed in cell free systems that do not assemble chromatin, but enhancers can stimulate promoters in cell free systems when the DNA is packaged into chromatin (Paranjape et al. 1994; Sheridan et al. 1995). Nucleosome assembly can interfere with the activity of some, but not all, proteins that are required for initiation of transcription or DNA replication (Workman et al. 1991).

Although it appears that histone H1 and acetylation of core histone are involved in chromatin-mediated repression their relative contribution in the process is yet unclear . In particular, the role of H1 on general transcription activity is unknown. In-vitro binding of histone H1 to chromatin in stoichiometric conditions does not have repressive effects (Howe et al. 1998; Sandaltzopoulos et al. 1994). At the same time, in vivo overproduction of mouse histone variant H1(0) results in repression of transcript levels of all polymerase II genes tested. (Brown et al. 1997). It is therefore possible that the maternal H1 variant is responsible for promoter repression in 2 cell embryos. In later stages, repression could be maintained because the level of hyperacetylated H4 drops starting with the 4-cell embryos (Wiekowski et al. 1997).

VII. A Role for DNA replication in relieving promoter repression

Two types of repression have been observed in 2-cell embryos: the "reversible" repression described above that can be relieved by enhancers when DNA is injected into 2-cell embryos, and an "irreversible" repression that occurs when DNA is injected into either pronucleus of a normally developing 1-cell embryo, and the injected embryo then undergoes mitosis to form a 2-cell embryo. Under these conditions, the injected promoter or replication origin is "irreversibly" repressed in that its activity cannot be restored either with butyrate or enhancers (Wiekowski et al., 1993; Henery et al., 1995). This phenomenon is unique to the first cell cycle since plasmid DNA injected into 2-cell embryos undergo reversible repression whether or not the injected 2-cell embryos is arrested in S-phase or continues cell division to produce 4-cell and then 8-cell embryos. What happens to DNA between completion of S-phase in a 1-cell embryo and formation of a 2-cell embryo that results in "irreversible" repression of injected plasmid DNA, but not of endogenous cellular DNA? One possibility is that the injected DNA is lost to the cytoplasm when the pronucleus, but not a zygotic nucleus, undergoes mitosis. This does not appear to be the case since transplantation of the injected pronucleus to a 2-cell embryo that then undergoes mitosis exhibits reversible repression of the plasmid encoded reporter gene. Instead, we suggest that DNA in early 1-cell embryos is subjected to repression before enhancer activation factors become available when zygotic genes are expressed. Once repression has been established, DNA replication may be required in order to displace repressor (histones?) so that transcription factors can bind to promoter and enhancer sequences (Wolffe 1991) (**Fig. 2**). Plasmid expression vectors, such as the ones used in these experiments, do not replicate when injected into mouse embryos (DePamphilis 1997), presumably because they lack a suitable replication origin. However, the 1-cell embryo's genome undergoes at least one round of replication prior to any zygotic gene transcription, an event that may be required to restore the zygotic genome to a transcriptionally competent state. DNA in 2-cell embryos competes for binding to both repressor and enhancer specific proteins (**Fig. 2**); the result of this competition determines the fraction of molecules that are transcriptionally active. Thus, enhancer stimulated promoter activity is greater when plasmid DNA is injected into late 2-cell embryos that progress to the 4-cell stage than in early 2-cell embryos that are arrested in S-phase (Henery et al. 1995) (**Table 1**).

In a recent paper Forlani et al. (Forlani et al. 1998) found that when plasmid DNA containing the intronic sequences of the human HPRT gene as an enhancer element attached to a "Pytk" promoter driving a reporter gene is microinjected in 1-cell embryos arrested by aphidicolin at a time that corresponds to post-DNA replication, the HPRT sequence can stimulate the "Pytk" promoter 4-5 fold as compared to the microinjected construct without the HPRT sequence. This observation led them to propose that acquisition of enhancer function requires the first round of DNA replication. However, the "Pytk" promoter activity seen in
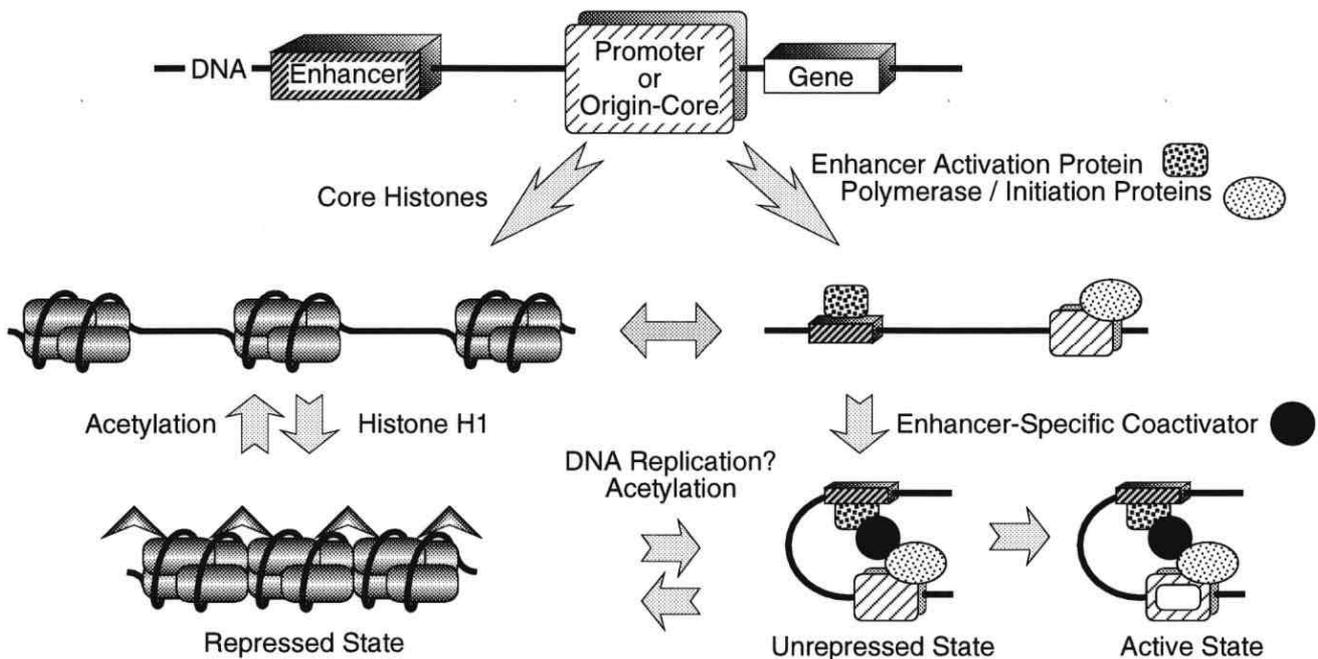
Figure 2. A working model showing the repression of promoters and replication origins by chromatin structure and the role of enhancers. Core histones and transcription/replication proteins (including enhancer activation proteins) compete to bind to the plasmid DNA microinjected into mouse oocytes and early embryos. Depending on their relative affinities for DNA, there is a dynamic equilibrium between DNA bound to core histones and DNA bound to various transcription/replication factors. In the presence of histone H1, DNA bound to core histones can then interact with them, resulting in a condensed chromatin structure and a repressed state. The repressive action of histone H1 can be blocked by acetylation of core histones. Sodium butyrate or trichostatin are known to inhibit histone deacetylases, and thus increase the fraction of acetylated core histones, causing stimulated transcription. On the other hand, the equilibrium can be shifted to the other direction where DNA bound to transcription/replication factors and enhancer activation proteins can interact with the enhancer specific coactivator resulting in the prevention of repression and the formation of an active state. DNA replication at each cell division may provide the cell with a chance to re-establish the equilibrium between repressed and unrepressed states.

1-cell embryos arrested after DNA replication is similar in magnitude, when compared to promoter activity seen in 1-cell embryos arrested before DNA replication. Thus, the low level of the HPRT sequence dependent stimulation appears not to be due to the release of promoter repression (primary role of enhancer function), but perhaps due to other secondary roles of enhancers as discussed above. This observation could also be explained by the effect of the additional transcription factor binding sites provided by the HPRT sequence on the promoter strength. Furthermore, it is also interesting to note that the experiments described in this paper use "Pytk" promoter that contains the tk promoter and the polyoma F101 enhancer (Py). Thus the Pytk promoter possibly represents not the promoter activity alone but the combined effect of promoter and enhancer activities. Thus, taken together, these results indicate that zygotic gene expression in 2-cell embryos and not the first round of DNA replication

is necessary for the acquisition of the ability to utilize enhancers in mammals, suggesting that one or more enhancer activation proteins, like the enhancer specific coactivator, is produced at this time.

VIII. The lack of enhancer function is unique to oocytes and fertilized eggs

The lack of enhancer function in oocytes raises the question whether the absence of enhancer function is a unique property of oocytes or a general property of other terminally differentiated cells. To explore this question, transcription activity was examined in terminally differentiated hNT neurons that ceased cell-division like oocytes, and their precursor, undifferentiated NT2 stem cells. The results showed that both NT2 and hNT cells could utilize Gal4VP16- and Sp1-dependent enhancers as well as

promoters (Lawinger et al. 1998). Thus, the lack of enhancer specific coactivator activity, and the corresponding lack of enhancer function, appears to be unique to oocytes and fertilized eggs, suggesting that it provides a safeguard against premature activation of genes prior to ZGE.

How does chromatin mediated repression and enhancer utilization help to regulate gene expression at the beginning of mammalian development? The onset of transcription during mouse development is regulated by a time dependent mechanism (zygotic clock), and takes place about 40 hours postfertilization, a time when a normally developing embryo is at the 2-cell stage. This stage of development also coincides with the onset of major chromatin repression of promoters (Majumder and DePamphilis, 1995). The paternal genome in sperm comes with protamines, whereas the maternal genome in eggs comes with a normal complement of core histones (Zirkin et al., 1989; Nonchev and Tsanev, 1990). After fertilization, they undergo chromatin remodeling to establish the zygotic genome at the 2-cell stage. This process of remodeling probably generates DNA that is not complexed with either histones or protamines (Rodman et al., 1981), and exposes promoters to transcription factors. Thus the zygotic clock may provide a mechanism to ensure no spurious transcription during the remodeling period. On the other hand, after the zygotic remodeling, the chromatin mediated repression of most promoters in 2-cell embryos may provide a mechanism for enhancer-mediated tissue specific transcription of genes during development and growth. Delaying expression of the enhancer specific coactivator prior to zygotic gene expression provides an additional mechanism for preventing inappropriate transcription of genes destined for expression in specific cell types.

The same mechanisms of transcriptional control that initiates mouse development also seem to occur in other animals. In mammals other than mice, transcription is delayed until the 2-cell or 16-cell stage, presumably by the same zygotic clock mechanism. Thus, the zygotic gene expression begins at the 2-cell stage in hamsters, the 4-cell stage in pigs, the 4- to 8-cell stage in humans, and the 8- to 16-cell stage in sheep, rabbits and cows (Telford et al., 1990; Seshagiri et al., 1992; Schulz, 1993). Whether or not repression of promoter activities appears at the 2-cell stage in these mammals, or is delayed until the same stage that transcription begins, remains to be seen. The S-phase of a 2-cell mouse embryo appears equivalent to the 6th cleavage stage in *Xenopus* where synthesis of heterogeneous, non-ribosomal mRNA is first detected. 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 (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. Furthermore, analogous stage-specific acquisition of specific transcriptional coactivators for enhancer function may also occur at the MBT (Xu et al., 1994).

Acknowledgements

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